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Title
Homodimerization of glucocorticoid receptor from single cells investigated using fluorescence correlation spectroscopy and microwells

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Abstract

Glucocorticoid receptor α (GR) binds to the promoter regions of target genes as a homodimer and activates its transcriptional process. Though the homodimerization is thought to be the initial and essential process, the dissociation constant for homodimerization of GR remains controversial. To quantify homodimerization of EGFP-GR, the particle brightness in lysates from single cell was estimated for the fraction of homodimeric EGFP-GR using fluorescence correlation spectroscopy and microwells. Fitting the data with a bimolecular reaction model, the dissociation constant was determined. Moreover slow-diffusion complex was observed. These results suggest that EGFP-GR forms not only a monomer-dimer equivalent state but also a large-molecular-weight complex.

Keywords
Dissociation constant, Fluorescence correlation spectroscopy, Glucocorticoid receptor, Homodimerization, Microwell, Single cell

Abbreviations
1. Introduction

Human glucocorticoid receptor α (GR) belongs to the nuclear receptor superfamily, which is associated with many physiological processes such as immune regulation, metabolism and so on [1]. It is known that GR binds to glucocorticoid response elements (GRE) in the promoter regions of target genes and regulates the transcription process [2-4]. Importantly, two GREs are aligned in a palindromic sequence on the genome and GR can bind to the palindromic sequence as a homodimer [5,6]. Therefore, its homodimerization property is important for the control of the GR transcriptional activity. However, the dissociation constant of GR homodimerization remains controversial [7,8]. One report indicated that the in vitro dissociation constant of GR homodimerization was 3.9 nM and concluded that almost all GRs dimerize in the absence of specific binding to GRE [7]. In contrast, another report indicated that the dissociation constant for homodimerization of GR was above 100 µM and that GR dimerizes on specific-binding GRE [8].

To determine the dissociation constant for homodimerization of GR in vitro at the single cell level, the ratio of the homodimer to the monomer of GR was calculated using the transient expression condition and a single-cell method combining fluorescence correlation spectroscopy (FCS) and polydimethylsiloxane (PDMS) chips of microwells (FCS-microwell system). FCS provides important information about the apparent number of particles and the diffusion time in a small detection volume [9, 10]. The apparent particle brightness, another obtained parameter, is affected by the fraction of the monomers and homodimers of fluorescent particles. Moreover, the
microwell system provides a stable condition for isolating protein molecules extracted from single cell [11]. There is no fluid exchange between individual microwells without any insulation material used at the interface between the coverslip and the microwells. Transient transfection was used for expressing a different concentration of EGFP-fused GR (EGFP-GR) in each cell. Combination of these techniques and conditions allows us to determine the concentration of fluorescently-labeled protein from single cells expressing different concentrations of EGFP-GR. The lower limit of the quantitative concentration of EGFP in the cell lysate in the microwells after cell lysis was investigated to confirm the feasibility of using the FCS-microwell system in this study.

Here, we report the dissociation constant for homodimerization of EGFP-GR in vitro determined using this FCS-microwell system. Moreover, slow diffusion of EGFP-GR was detected compared with the theoretical value calculated from the molecular weight of homodimeric EGFP-GR. Our results suggest that EGFP-GR forms a monomer-dimer equivalent state and a large-molecular-weight complex in microwells.

2. Materials and methods

2.1. Constructs and chemicals

The expression vectors for enhanced green fluorescent protein (EGFP) fused with human glucocorticoid receptor α (EGFP-GR) were described previously [12]. A synthetic ligand of GR, dexamethasone (Dex), was purchased from Sigma-Aldrich. Dex was used at a concentration of 0.5 µM in phenol red-free
medium (opti-MEM, GIBCO) for activation of GR. The components of the lysis buffer were 80% CelLytic M Cell Lysis Reagent (Sigma-Aldrich), 10 mM MgCl₂, 0.1% SDS and 200 U/mL Benzonase nuclease (Sigma-Aldrich).

2.2. Single-cell method combining FCS and microwells

The microwells were 60 µm in diameter and 40 µm in depth, with a volume of 113 pL (Fig. 1A). Each microwell was denoted by a number and a letter to determine the positions of microwells of interest and find them easily after Dex treatment and 20 min incubation. The polydimethylsiloxane (PDMS) chip with microwells was originally designed and ordered to Fluidware Technologies Inc., Tokyo, Japan. The microwells and coverslips (No. S1; Matsunami Glass, Tokyo, Japan) were treated with N101 blocking reagent (Nippon Oil and Fats, Tokyo, Japan) to prevent adsorption of proteins.

Schematic diagrams of the single-cell method combining FCS and PDMS chips with microwells (FCS-microwell system) are shown in Fig. 1B and C. The PDMS chip was attached to a glass stick with double-sided tape (Nitoms, Tokyo, Japan) and the chip was pressed onto a coverslip in opti-MEM (Fig. 1B, left). After pressing the PDMS chip onto the coverslip, the positions (a number and a letter) of the microwells in which single cell was cultured were noted down (Fig. 1B, right). The medium on the coverslip was changed to lysis buffer and the protein extracted from each cell was kept in the microwell after cell lysis, following which FCS measurements were carried out in each microwell (Fig. 1C).
2.3. Cell culture and transfection in microwells

HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) at 37 degrees with 5% CO₂. After transfection of EGFP-GR, DMEM with charcoal-stripped 10% FBS was used as the culture medium to prevent activation of GR by endogenous ligands in FBS. To culture the HeLa cells, the microwells on the PDMS chips were washed using a detergent water, which was then evaporated in a glass vacuum dryer to remove the water from the microwells. After evaporation, the cell culture medium containing the HeLa cell suspension was poured onto the PDMS chip in a 60 mm dish and incubated for 4 hours at 37 degrees.

HeLa cells on 35 mm dishes were transfected using Optifect reagent (Invitrogen). After replacement of the culture medium on dishes with fresh medium, 0.1 µg of plasmid-encoded EGFP or 3.0 µg of plasmid-encoded EGFP-GR was mixed with Optifect in opti-MEM and added to the cell culture dishes. After 24-hour incubation at 37 degrees with 5% CO₂, the transfected HeLa cells were trypsinized and transferred to the microwells on the PDMS chips. Then the expression level of EGFP-GR was analyzed by Western blotting and found to be 7.07-fold higher than that of endogenous GR in the living HeLa cell (Supplemental method and Fig. S1).

2.4. LSM imaging and FCS measurements.

LSM imaging and FCS measurements were performed using an
LSM510-ConfoCor2 (Carl Zeiss, Jena, Germany) equipped with an Ar ion laser, water immersion objective (C-Apochromat, 40X, 1.2N.A., Corr; Carl Zeiss, Jena, Germany), a photomultiplier for LSM imaging and an avalanche photodiode detector for FCS measurements. The pinhole diameter was adjusted to 70 µm. EGFP was excited at 488 nm and the EGFP fluorescent signals were detected above 505 nm for LSM imaging and at 505-550 nm for FCS measurements. FCS measurement was carried out five times for 10 seconds.

2.5. Data analysis of FCS measurements

Data obtained from FCS measurements were calculated with AIM software (Carl Zeiss, Jena, Germany). The autocorrelation function, \( G(\tau) \) was defined as follows:

\[
G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}
\]  

(1)

where \( \tau \) shows the delay time, \( I \) is fluorescence intensity and \( G(\tau) \) denotes the autocorrelation function, respectively. The obtained autocorrelation functions were fitted using a one-component model as follows:

\[
G(\tau) = 1 + \left( 1 + \frac{F_{\text{triplet}} e^{-\tau/\tau_{\text{triplet}}}}{1 - F_{\text{triplet}}} \right) \cdot \frac{1}{N} \left( 1 + \frac{\tau}{\tau_D} \right)^{-1} \cdot \left( 1 + \frac{1}{\frac{s^2}{\tau_D}} \right)^{-\frac{1}{2}}
\]  

(2)

where \( F_{\text{triplet}} \) is the average fraction of triplet state molecules, \( \tau_{\text{triplet}} \) is the average relaxation time and \( \tau_D \) is the average diffusion time of molecules. The diffusion constant of EGFP-GR was calculated from the diffusion constant of a
standard molecule, rhodamine 6G ($D_{R6G}$; 414 $\mu$m²/s) [13] and the ratio of diffusion times $\tau_{R6G}$ and $\tau_{EGFP-GR}$. $N$ is the average number of fluorescent molecules in the effective observation volume ($V_{eff}$) defined by 3D Gaussian volume elements with lateral radius $w_0$ and axial radius $z_0$. $s$ shows the structure parameter representing the ratio of $w_0$ to $z_0$ ($s=z_0/w_0$). $w_0$ and $z_0$ were determined by calibration measurement of R6G.

$$w_0 = \sqrt{4D_{R6G}\cdot\tau_{R6G}}$$

-(3)

$$s = \frac{z_0}{w_0}$$

-(4)

The effective observation volume was calculated using the following equation.

$$V_{eff} = \frac{3}{2} \cdot \pi^2 \cdot w_0^2 \cdot z_0$$

-(5)

The fitting models of a one-component with normal or anomalous diffusion and two-component with normal diffusion for FCS measurement in the cells that expressed EGFP were compared (Fig. S2). There were no significant differences in the chi square values between the autocorrelation function and fitting curves and number of molecules, even if EGFP was present in the cell. Moreover, the number of molecules ($N$) was not different in the models and this indicates estimated dissociation constant is the same among these models. Therefore, the fitting model of a one-component with normal diffusion was selected for fitting to all data.
To remove the effect of background fluorescence on the obtained $N$, the corrected $N (N_{\text{corr}})$ was calculated as follows [14,15]:

$$N_{\text{corr}} = \frac{N_{\text{meas}} \cdot (I_{\text{meas}} - I_B)^2}{(I_{\text{meas}})^2}$$  \hspace{1cm} (6)

where $N_{\text{meas}}$ is the number of molecules obtained from FCS measurements, $I_{\text{meas}}$ is the measured average fluorescent intensity and $I_B$ is the background average fluorescent intensity from FCS measurement of a non-transfected HeLa cell lysate.

The concentration of fluorescent molecules ($[C_{\text{corr}}]$) was calculated from the effective observation volume ($V_{\text{eff}}$), corrected number of molecules ($N_{\text{corr}}$) and Avogadro’s number ($N_A$) as given below.

$$[C_{\text{corr}}] = \frac{N_{\text{corr}}}{V_{\text{eff}} \cdot N_A}$$  \hspace{1cm} (7)

### 2.6. Determination of the lower limit of the EGFP concentration by FCS

EGFP was expressed in Escherichia coli (BL21 [DE3]) and purified using a Ni affinity column (GE Healthcare). An equivalent-single-cell-lysate solution (ESS), defined as which contained the same concentration of endogenous protein extracted from a single cell in a microwell (single cell in 113 pL), was prepared to measure the background fluorescence. The number of cells was estimated using a cell counter, and then the volume of the lysis buffer was calculated by multiplying of number of cells by the microwell volume (113 pL). EGFP diluted with the ESS was measured by FCS in three microwells and the counts per particle (CPP) in which the averaged fluorescent intensity was
divided by the number of molecules \((N)\), were obtained for different concentrations of EGFP. For the same laser intensity, the average number of molecules and the average fluorescent intensity are linearly related. Therefore, the measured CPP should be constant if the fluorescent intensity and the number of EGFP molecules are correctly determined. The CPP value with the lowest relative standard deviation (LRS) determined from five independent measurements yielded an expected \(\text{CPP}_{\text{LRS}}\) value of 8.71 kHz (blue dashed line in Fig. 2B). The total deviation between \(\text{CPP}_{\text{LRS}}\) and measured CPP values \((X^2)\) was calculated for each EGFP concentration according to the following equation:

\[
X_j^2 = \sum_{i=1}^{5} \frac{(\text{CPP}_{\text{LRS}} - \text{CPP}_{j,i})^2}{\text{CPP}_{\text{LRS}}}
\]  

where \(i = 1 - 5\) denotes independent measurements for the same concentration \((j)\). Hence, \(X^2\) shows the sum of the squared deviation normalized to \(\text{CPP}_{\text{LRS}}\). If the total deviation between \(\text{CPP}_{\text{LRS}}\) and the measured CPP values \((X^2)\) is smaller than 9.488 \((P = 0.05\) and the degree-of-freedom = 4), the average CPP value is statistically similar to \(\text{CPP}_{\text{LRS}}\) (Fig. 2B). The statistically similar point at the lowest concentration of EGFP was defined as the lower limit for determination of the EGFP concentration (Fig. 2C).

### 2.7. Comparison of cell volume measurements between the Z-stack imaging method and FCS-microwell system

To confirm whether the extracted EGFP was conserved in the microwells,
the cell volumes calculated from the Z-stack image of LSM ($V_{\text{cell-Z}}$) and FCS-microwell system ($V_{\text{cell-F}}$) were compared. An optical slice of $512 \times 512$ pixels in the Z-stack image was acquired. The fluorescence-detected voxel number in the Z-stack image was counted using Image J software (NIH) and the cell volume from Z-stack image ($V_{\text{cell-Z}}$) was calculated by multiplying the voxel number by the size ($0.2 \ \mu m \times 0.2 \ \mu m \times 0.87 \ \mu m$) used in the objective (C-Apochromat, 40X, 1.2N.A., Corr).

Moreover, cell volume could be calculated using the FCS-microwell system. If the EGFP-containing lysate remains in the microwell after cell lysis, the total concentration of EGFP in the single cell before lysis and that in the microwell after lysis can be represented by the following equation.

\[
[C_{\text{cell,corr}}] \cdot V_{\text{cell-F}} = [C_{m, \text{corr}}] \cdot V_m
\]  

-(9)

where $V_{\text{cell-F}}$ and $V_m$ are the volumes of the cell from the FCS-microwell system and microwell (113 pL), respectively. The EGFP concentration inside the cell before lysis ($[C_{\text{cell,corr}}]$) and that in the microwell after lysis ($[C_{m, \text{corr}}]$) were obtained by FCS measurement inside the HeLa cell before cell lysis and in the microwell after cell lysis in the same microwell, respectively. The cell volume was calculated using the following equation:

\[
V_{\text{cell-F}} = \frac{[C_{m, \text{corr}}] \cdot V_m}{[C_{\text{cell,corr}}]}
\]  

-(10)

2.8. Determination of dissociation constant of EGFP-GR in homodimerization

The dissociation constant $K_d$ for homodimerization of EGFP-GR was determined using the following equations:
where \([M_0]\) is the total concentration of EGFP-GR in the microwell, and \([M]\) and \([D]\) are the concentrations of monomeric and homodimeric EGFP-GR, respectively.

To obtain the \([M]\) and \([D]\), the monomeric fraction \(F_m\) and homodimeric fraction \(F_d\) \((F_m + F_d = 1)\) of EGFP-GR were calculated using equations (13), (14) and (15) \([16]\). FCS measurement can be used to obtain the apparent number of molecules and brightness, which is defined as the counts per particle (CPP). When monomeric and homodimeric EGFP-GR are contained in the lysate, the apparent CPP \((CPP_{\text{EGFP-GR, app}})\) is obtained as shown by the following equation \([16]\).

\[
CPP_{\text{EGFP-GR, app}} = \frac{F_m \cdot \eta_m^2 + F_d \cdot \eta_d^2}{F_m \cdot \eta_m + F_d \cdot \eta_d}
\]

where \(\eta_m\) and \(\eta_d\) are the CPP of monomeric and homodimeric EGFP-GR, respectively.

If the CPP of the tandem dimer of EGFP is twice that of EGFP (Fig. S3), the CPP of homodimeric EGFP-GR \((\eta_d)\) will be twice the CPP of monomeric EGFP-GR \((\eta_m)\), which is the same as that of EGFP \((CPP_{\text{EGFP}})\).

\[
\eta_m = CPP_{\text{EGFP}}
\]

\[
\eta_d = 2 \cdot CPP_{\text{EGFP}}
\]

By using equations (13), (14) and (15), the fractions of monomeric and homodimeric EGFP-GR \((F_m\) and \(F_d\)\) are shown as equations (16) and (17).
where,

\[ R = \frac{\text{CPP}_{\text{EGFP, GR, app}}}{\text{CPP}_{\text{EGFP}}} \]  

\[ F_m = \frac{4 - 2R}{3 - R} \]  

\[ F_d = \frac{R - 1}{3 - R} \]

3. Results

3.1. Lower limit for determination of EGFP concentration

To quantify the number of EGFP-tagged target proteins in a single cell, FCS measurements were performed with equivalent-single-cell-lysate solution (ESS)-diluted EGFP in the microwell for determination of the lower limit of the EGFP concentration. FCS measurements were performed in three microwells and similar autocorrelation functions and fit residuals were obtained for all three (Fig. 2A).

To clarify the lower limit for determination of the EGFP concentration, counts per particle (CPP\text{EGFP}) was calculated, because CPP\text{EGFP} should be constant when the EGFP fluorescent intensity and number of molecules are measured correctly. Without background correction, the CPP\text{EGFP} was reduced with decreasing concentrations of EGFP (Fig. 2B). On the other hand, the CPP\text{EGFP} was constant until the concentration of 0.17 nM with background correction. The total deviation between CPP\text{LRS} (8.71 kHz) and the measured CPP (X\text{2} in
eq. (8)) was calculated for each concentration (Fig. 2C). When the total deviation was lower than the dashed blue line (9.488; P = 0.05 and degree-of-freedom = 4), CPP\textsubscript{EGFP} was similar to CPP\textsubscript{LRS} (Fig. 2B). The lower limits of the concentrations of EGFP without and with background correction were 2.5 nM and 0.17 nM, respectively, as shown by the black arrows in Fig. 2D. These results indicated that the EGFP concentration from 0.17 to 1741 nM could be quantified by using the FCS-microwell system with background correction.

3.2. Comparison of cell volume between FCS-microwell system and Z-stack image method.

The cell volumes obtained from Z-stack image and the FCS-microwell system were compared. Typical images of an EGFP-expressing HeLa cell are shown in Fig. 3A and autocorrelation functions and fit residuals before and after cell lysis in Fig. 3B. Scatter plots were obtained and linear fitting with Deming regression (Y = 1.19X-0.69; slope; 1.19±0.32, intercept; -0.69±1.15) was performed for all single-cell data obtained from Z-stack image method and the FCS-microwell system method (Fig. 3C). The cell volumes calculated by both methods agreed well with each other because the plot was on a line with a slope of almost 1.0. This indicated complete lysis of the cell in microwells and that there was no leakage from the microwell system as well as that the concentration of EGFP in the microwells after lysis could be determined correctly.
3.3. Determination of dissociation constant of EGFP-GR in homodimerization

The amounts of homodimeric EGFP-GR were determined using the FCS-microwell system. Typical images of a HeLa cell expressing EGFP-GR are shown in Fig. 4A. EGFP-GR localized in the nucleus after addition of dexamethasone (Dex). The extraction efficiency of EGFP-GR was 90% and reached a plateau at 90 min after cell lysis treatment (Fig. S4). FCS measurements were performed in microwells after 90-min cell-lysis treatment and typical autocorrelation functions and fit residuals of EGFP and EGFP-GR in the absence and presence of Dex are shown in Fig. 4B. The CPP was considered for distinguishing the fractions of monomeric and homodimeric EGFP-GR because the change of CPP between monomeric and homodimeric EGFP-GR is more sensitive than that of the diffusion time of EGFP-GR. The normalized CPP increased with the increase in the total concentration of EGFP-GR in the presence of Dex. On the other hand, it was constant in the absence of Dex (Fig. 4C). The homodimeric fraction of EGFP-GR was calculated from the normalized CPP using equation (17) (Fig. S5). Next, the concentration of homodimeric EGFP-GR was calculated using the homodimeric fraction and EGFP-GR concentration. The data were fitted by the bimolecular reaction model (equation (11)) using the curve fitting based on the non-linear least-squares in whole range. The dissociation constant for homodimerization of GR was determined to be 49.6±7.27 nM (Fig. 4D, Blue dashed line). In order to small number of data points at higher concentration, the data were fitted by the different method, the weighted non-linear
least-squares in whole range. The weighting factor of the each data point for fitting was the inverse of the number of data points within the window of ± 2.5 nM from each data point. However, the dissociation constant which was obtained to be 34.3±5.37 nM was similar to that by least-squares fitting in whole range (Fig. S6).

Next, the data points in the 0 to 50 nM range were fitted by least-squares fitting. The maximum amount of endogenous GR in COS-1 is 61 nM based on the previously reported 111000 molecules per cell (1420 fmol/mg) [17] and our finding of a 3 pL cell volume. Moreover, 16200 fmol/mg in cytotrophoblasts [18] was calculated to be 701 nM using the same method. The range above a total EGFP-GR concentration of 50 nM in the microwell was ignored in this fitting model because the 50 nM total concentration of EGFP-GR corresponded to a 1.5 µM concentration in the cell calculated using the 3 pL cell volume, and a concentration of over 1.5 µM in a cell seemed to be overexpression compared with the endogenous concentration of GR. The dissociation constant for homodimerization of EGFP-GR was determined to be 107±19 nM and 49.6±7.27 nM using the least-squares fitting in the range of 0 to 50 nM and whole range, respectively (Fig. 4D). The dissociation constant suggested that all GR did not consist of a homodimer but that there was equilibrium between the monomer and homodimer forms in both fitting.

Moreover, the diffusion constant determined from the autocorrelation function of FCS measurements was lower than the theoretical diffusion constant calculated from the molecular weight of homodimeric EGFP-GR (240 kDa) (Fig. 4E). These results suggested that EGFP-GR formed not only a
monomer-dimer equivalent state, but also a large-molecular-weight complex with interacting proteins. This lower diffusion constant might originate from GR-DNA complexes but this probability is small because a nuclease (Benzonase) was present in the extraction buffer.

4. Discussion

In this study, we employed an in vitro single-cell method combining FCS and microwells in a PDMS chip (FCS-microwell system). There are many applications of FCS for protein dynamics in single living cells [19,20]; however, the total amount of functional protein in a single cell is difficult to estimate because of photobleaching of fluorescent proteins and their heterogenous distribution. In contrast, the FCS-microwell system can be used to isolate the cell lysate from a single cell and total amount of functional protein can be determined without photobleaching. The quantitative range of the EGFP concentration was from 0.17 nM to 1741 nM with background correction (Fig. 2D). This suggested that the FCS-microwell system could be used to quantify the amounts of functional protein tagged EGFP from 6.4 nM to 65.6 µM concentrations in single cell. However, it should be noted that this limitation was defined under the condition of 5 measurements for 10 seconds and that the excitation with lower laser power may enable the quantification in the higher concentration region above 1741 nM.

Moreover, the cell volumes determined by the Z-stack image method and FCS-microwell system method were in good agreement (Fig. 3C). The
FCS-microwell system is easier to use than the Z-stack image method for estimation of cell volume because only FCS measurements in the cell before lysis and in the microwell after lysis are required.

On the basis of the concentration of EGFP-GR in the microwell using FCS-microwell system, the dissociation constant of GR homodimerization determined to be 49.6 nM and 107 nM with two different fitting concentration range (Fig. 4D). There is two-folds difference between these dissociation constants. This result may indicate the different form of GR homodimerization at 0 to 50 nM and above 50 nM concentration range because of the presence and different effect of other components (such as cofactors). On consideration with endogenous concentration of GR in the cell, the dissociation constant of 107 nM might be more effective to the homodimerization in the cell. Moreover, the dissociation constant for homodimerization of GR suggested that GR dimerized partially in the absence of GRE because our experiments were performed without addition of GRE-containing oligonucleotides and with lysis buffer containing Benzonase nuclease.

The diffusion constant showed that GR formed a larger complex than dimeric GR (Fig. 4E). Previous studies have reported proteins interacting with GR such as Hsp90 and cofactors [21,22,23]. However, the regulatory proteins for homodimerization of GR remain unknown. We speculate that the large complex we detected contains interacting proteins and that these proteins regulate homodimerization in the living cell.

In this work, single-cell measurement was performed for homodimerization of GR and complex formation in single cells. The variance of the CPP and
diffusion constant from single cell were focused on to examine the heterogeneity of homodimerization and complex formation in single cells. The relative standard deviation of the normalized CPP and diffusion constant of EGFP-GR were calculated to be in the ranges from 0 to 10 nM, 10 to 20 nM and 20 to 30 nM (Fig. S7). These values were higher than those of the relative standard deviation of EGFP, corresponding to the measurement error (Fig. S7). These results suggested that the homodimerization and complex formation of EGFP-GR were heterogeneous in single cells. This may show that single-cell methods are needed for understanding the functions of GR. Recently, it was suggested that the ratio of monomers and dimers of GR does not necessarily change the transcriptional activity determined by comparison of Number and Brightness analysis in living cells and quantitative PCR using a whole cell lysate [24]. It may thus be necessary to determine the absolute expression levels of monomers and dimers to understand relationship between monomer/dimer and transcription activity. Here, the FCS-microwell system was used to determine the monomer and dimer concentrations at the single-cell level. This system could be revealed the relationship between the amount of homodimeric GR and its transcriptional activity. In addition to GR homodimer formation, the heterodimers of EGFP-GR and other nuclear receptors (like MR) should be considered in biological systems in the near future.

In conclusion, we established the FCS-microwell system for single-cell analysis and determined the dissociation constant for homodimerization of GR in vitro using this method. In addition, the relationship between the amount of homodimeric GR and its transcriptional activity could be determined using the
FCS-microwell system. This should be helpful to understand the mechanisms of the transcriptional activity of GR.

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Figure legends

Fig. 1 Schematic diagram of FCS-microwell measurement system.

(A) An overview of the PDMS chip (left) and an enlarged image of microwells on the PDMS chip (right). EGFP-expressing cells were cultured in microwells.  
(B) Schematic diagram of the single-cell isolation and an isolated single cell in a microwell, and (C) the lysis step for single cell and FCS measurements (scale bar: 20 µm).

Fig. 2 Lower limit for determination of the EGFP concentration.

(A) FCS measurement position, autocorrelation function and fit residual in three microwells with 200 nM recombinant EGFP. Black solid line, Microwell 1; Red dotted line, Microwell 2; Blue dashed line, Microwell 3; White cross, FCS measurement point. (Scale bar: 20 µm)  
(B) CPP with and without background correction of FCS-obtained data at each concentration. The average value and standard deviation were calculated from five measurements. Open symbol, without correction; Filled symbol, with correction; Blue dashed line, CPP_{LRS} (8.71 kHz).  
(C) Total deviation of CPP between 5 data determinations and CPP_{LRS} in (B) at each concentration. Open symbol, without correction; Filled symbol, with correction; Blue dashed line (9.488); P = 0.05, degree-of-freedom = 4; Black arrow, lower limit for determination of EGFP concentration.  
(D) FCS-obtained EGFP concentration at each concentration. The average value and standard deviation were calculated from five
measurements. Open symbol, without correction; Filled symbol, with correction; Blue dashed line, Linear line with slope being 1.0; Black arrow, lower limit for determination of EGFP concentrations: 2.5 nM and 0.17 nM without and with correction, respectively.

Fig. 3 Comparison of cell volume between FCS-microwell system and Z-stack image method.

(A) Typical images of EGFP-expressing cell in microwell before and after lysis (White cross, FCS measurement position; Scale bar, 20 µm). (B) Typical autocorrelation functions and fit residuals determined by FCS measurement in living cell and lysate. Black solid line, EGFP in living cell; Red dotted line, EGFP in lysate. (C) Cell volume determined using the FCS-microwell system (Vcell-F) and Z-stack image method (Vcell-Z). Date number was 21. Filled symbol, single-cell data; Red dashed line, linear fitting with Deming regression: Y = 1.19X - 0.69; Slope; 1.19 ± 0.32, Intercept; -0.69 ± 1.15.

Fig. 4 EGFP-GR dimerization and complex formation.

(A) Typical image of EGFP-GR expressing cell before and after Dex treatment and after lysis. EGFP-GR translocated to the nucleus by Dex treatment and was extracted into lysis buffer. White cross, FCS measurement point; Scale bar, 20 µm. (B) Typical autocorrelations and fit residuals of EGFP and EGFP-GR with and without Dex treatment. Black solid line, EGFP; Red dotted line, EGFP-GR without Dex treatment; Blue dashed line, EGFP-GR with Dex treatment. (C) CPP of EGFP-GR normalized by CPP of EGFP. Data numbers were 16 and
114 in the absence and presence of Dex, respectively. Open symbol, EGFP-GR without Dex treatment; Filled symbol, EGFP-GR with Dex treatment; Blue dashed line, EGFP. (D) Determination of the dissociation constant in EGFP-GR dimerization ($K_d$; 49.6 ± 7.27 nM and 107 ± 19 nM in the least-squares fitting in whole range and 0 to 50 nM range, respectively). Filled symbol, EGFP-GR with Dex treatment; Red solid line, least-squares fitting in the region of 0 to 50 nM; Blue dashed line, least-squares fitting in whole region by equation (11) in materials and methods. Data number was 114. (E) Diffusion constant of EGFP-GR with and without Dex treatment. Data numbers were 16 and 114 in the absence and presence of Dex, respectively. Open symbol, EGFP-GR without Dex treatment; Filled symbol, EGFP-GR with Dex treatment; Blue dashed line, theoretical diffusion constant of homo dimeric EGFP-GR calculated by molecular weight (240 kDa).
Fig. 1

(A) 10 mm

60 μm

40 μm

113 pL

(B) Glass stick
Double-sided tape
Microwell
Opti-MEM
Coverslip
Cell
Press

(C) Lysis buffer
Press

Normalized autocorrelation

**Normalized autocorrelation**

Ar laser (488 nm)

Dichroic mirror

Objective

EGFP (Fluorescence)
Fig. 2

(A) Image of microwells with labels 1, 2, and 3. 20 μm scale bar.

(B) Graph showing autocorrelation of CPP with respect to set concentration (nM) for Microwell 1, Microwell 2, and Microwell 3. 

(C) Graph showing fit residual of CPP with respect to time (μs) for Microwell 1, Microwell 2, and Microwell 3.

(D) Graph showing total deviation of CPP with respect to set concentration (nM) for EGFP without correction and EGFP with correction. The blue dashed line indicates P = 0.05.

(E) Graph showing FCS concentration (nM) vs. set concentration (nM) for EGFP without correction and EGFP with correction. The blue dashed line indicates a linear fit with a slope of 1.0.
Fig. 3

(A) Lysis
30 min
37 °C

(B) EGFP in living cell
EGFP in lysate

(C) Cell volume by
FCS-microwell system (pL)
Single cell (N = 21)
Linear fitting

Fit residual
Time (µs)

Normalized autocorrelation
Time (µs)
**Fig. 4**

(A) Dex treatment: 20 min at 37 °C followed by lysis: 90 min at 37 °C.

(B) Normalized autocorrelation vs. time (µs) for EGFP, EGFP-GR; Dex (-), and EGFP-GR; Dex (+).

(C) Normalized CPP of EGFP-GR vs. total EGFP-GR (nM) for EGFP, EGFP-GR; Dex (+), and EGFP-GR; Dex (-).

(D) Diffusion constant (µm²/s) vs. total EGFP-GR (nM) for least square fitting in 0 to 50 nM and whole region, along with theoretical value of dimeric EGFP-GR.

(E) Dimeric EGFP-GR (nM) vs. total EGFP-GR (nM) for least square fitting in 0 to 50 nM and whole region, along with theoretical value.