Determining the stoichiometry of protein heterocomplexes in living cells with fluorescence fluctuation spectroscopy

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Edited by Roger Y. Tsien, University of California at San Diego, La Jolla, CA, and approved December 20, 2006 (received for review July 31, 2006)

The brightness of fluorescence fluctuations provides information about protein interactions in the intercellular environment under equilibrium conditions. Here we demonstrate that the stoichiometry of a protein complex containing two proteins labeled with CFP and YFP can be determined by brightness analysis. The brightness profile, which characterizes the brightness as a function of the labeled protein coexpression ratio, together with brightness titration experiments, provides sufficient information to quantify the composition of a protein complex under stoichiometric binding conditions. The selective and simultaneous excitation of proteins labeled with CFP and YFP by choosing different excitation wavelengths is used to identify the composition of the protein complex. Interactions between nuclear receptors and their coactivators play a crucial role in the regulation of gene expression. We choose the ligand-binding domain of retinoic X receptor and the nuclear receptor interacting domain of the steroid receptor coactivator-1 as a model for exploring the formation of a hetero-oligomer by brightness analysis directly in living cells. Our results show the formation of a heterotetramer with three nuclear receptors binding to the coactivator domain. Elimination of one of the nuclear receptor binding sites through a truncation mutant changed the interaction between both proteins significantly and led to a nuclear receptor-induced oligomerization of the truncated coactivator. Quantifying protein interactions in a cell is an important step in understanding cellular function on a molecular level. This study provides proof-of-principle experiments that illustrate the potential of brightness analysis as a powerful tool for quantifying protein interactions in living cells.

coactivator | fluorescence correlation spectroscopy | protein interactions | two-photon

Proteins in a cell interact and form complexes that perform biological functions. The ready availability of fluorescent proteins as markers for cellular proteins has opened up the possibility to observe protein interactions in real time by fluorescence methods. Fluorescence resonance energy transfer (FRET) is the most widely used technique for detecting protein interactions. However, FRET typically identifies the presence of protein interactions without providing quantitative information about the protein complex. Here, we describe a method based on fluorescence fluctuation spectroscopy (FFS) with the ability to quantify the stoichiometry of protein complexes in living cells.

FFS is an attractive candidate for studying protein interactions in cells and is based on fluctuations in the fluorescence intensity observed in a small observation volume (<1 fl) that are due to individual proteins entering or leaving the volume (1). Fluorescence correlation spectroscopy (FCS) uses correlation functions to determine the concentration and temporal properties of proteins, and dual-color FCS has been used to detect protein interactions in cells (2, 3). However, a quantitative characterization of protein interactions has proven to be difficult. We argued previously that brightness analysis of fluctuations is a promising method for quantifying protein interactions in living cells (4). The brightness of a molecule is defined as the average fluorescence intensity of a single particle, and analysis methods that determine the brightness from fluctuation data are available (5, 6).

Brightness encodes the stoichiometry of protein complexes. Consider the case of a dimer. If the monomeric protein carries a fluorescent protein of brightness ε then the homodimer exhibits a brightness of 2ε, because it carries two fluorophores. This concept was experimentally verified by using GFP as a marker and applied to study the concentration-dependent dimerization of nuclear receptors (NRs) in living cells (7). However, the use of a single fluorescent color limits brightness analysis to homocomplex formation. Two fluorescent colors are required to tackle heterointeractions. We developed the analysis tools for brightness analysis of two colors and tested it by using the heterodimer of the ligand-binding domains (LBD) of the NRs retinoic X receptor (RXR) and retinoic acid receptor (RAR) in CV-1 cells (8). Quantifying heterointeractions is considerably more complex than the analysis of homocomplex formation, and a method that identifies a heterocomplex of unknown composition is currently unavailable. In this manuscript we devise a methodology that solves this problem and identifies the stoichiometry of two proteins labeled with CFP and YFP within a cellular protein complex. We also introduce the brightness profile, which characterizes the normalized brightness as a function of the coexpression ratio of the labeled proteins. The brightness profile is used together with brightness titration experiments to deduce the composition of a protein complex at stoichiometric binding conditions. We illustrate the method using a protein complex that is larger than a dimer to highlight the inherent strength of brightness analysis.

NRs such as RXR are known to interact with coactivators. It is generally thought that two NRs bind a single coactivator molecule, thus providing a suitable test system that goes beyond the simple dimer model. We choose the LBD of RXR and the NR interacting domain (NID) of SRC1 (steroid receptor coactivator 1) as a minimal model for exploring the formation of a hetero-oligomer by FFS directly in living cells. Brightness analysis revealed that RXRLBD and SRC1NID form a heterotetramer with three RXRLBDs binding to one SRC1NID molecule. SRC1NID contains three NR boxes (see Fig. 1) that have been identified as the binding motifs involved in the recruitment of NRs (9–11). Elimination of one of the NR boxes through a

Author contributions: Y.C. and J.D.M. designed research; Y.C. performed research; Y.C. and J.D.M. analyzed data; and J.D.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: FFS, fluorescence fluctuation spectroscopy; RXR, retinoic X receptor; LBD, ligand-binding domain; SRC1, steroid receptor coactivator 1; NR, nuclear receptor; NID, NR interaction domain; 9cRA, 9-*cis*-retinoic acid.

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This article contains supporting information online at www.pnas.org/cgi/content/full/0606557104/DC1.

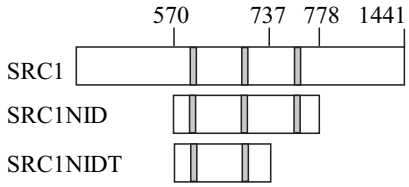

Fig. 1. Schematic diagram of the SRC1 gene, its NID SRC1NID (amino acid residues: 570–778), and its truncated fragment SRC1NIDT (amino acid residues: 570–737) containing only two of the three NR boxes. The NR boxes are shown as shaded regions.

truncation mutant resulted in a NR-mediated association of coactivator fragments. This result indicates the importance of protein conformation in the interaction between coactivators and NRs. Our brightness study serves as a proof-of-principle that illustrates the promising potential of brightness analysis for quantifying protein interactions in living cells to a degree not previously achieved.

Theory

A few parameters and equations that are needed later are introduced. Assume that two proteins, A-CFP and B-YFP, are able to interact with themselves and one another. For simplicity we introduce the model $xA_i + yB_j \leftrightarrow zA_rB_s$, where we drop the CFP and YFP label for convenience. Each protein is able to associate into the homocomplexes A_i and B_j, and upon interaction the heterocomplex A_rB_s is formed. More general models are conceivable, but this particular example will be used later. The total concentration of proteins A and B expressed in monomers is $C(A_M) = iC(A_i) + rC(A_rB_s)$ and $C(B_M) = jC(B_j) + sC(A_rB_s)$, respectively. The concentration $C(x)$ of species x is proportional to the occupation number $N(x)$ measured by FFS. The average photon count rate of the sample is related to the brightness and occupation number of the species A and B, $\langle k\rangle = \varepsilon_A N(A_M) + \varepsilon_B N(B_M)$ (12). The brightness ε_M of monomeric A or B is identical when excited at 902 nm for our experimental setup, $\varepsilon_M = \varepsilon_A = \varepsilon_B$, and the photon count rate reduces to $\langle k\rangle = \varepsilon_M(N(A_M) + N(B_M))$. Another experimental parameter available is the coexpression ratio $r_{AB} = N(A_M)/N(B_M)$ of the proteins A and B (8). Knowledge of ε_M, $\langle k\rangle$, and r_{AB} determines the concentration of protein A expressed in monomer units as $N(A_M) = \langle k\rangle/(\varepsilon_M(r_{AB} + 1))$. Similarly, the concentration of protein B expressed in monomer units is given by $N(B_M) = r_{AB}N(A_M)$.

Let us assume for now that the brightness of a protein complex is ε. It is convenient to introduce the normalized brightness as the ratio of the measured brightness to the brightness of a monomer, $\eta = \varepsilon/\varepsilon_M$. The normalized brightness simplifies the comparison of data taken under different experimental conditions, where the absolute brightnesses vary. The normalized brightness of a mixture of species is given by a combination of $\eta(X_i)$ and the population fraction $f(X_i) = N(X_i)/\Sigma_i N(X_i)$ of each species X_i (2). For the three species A_i, B_j, and A_rB_s, the normalized brightness of a mixture is given by

$$\eta = \frac{f(A_i)\eta(A_i)^2 + f(B_j)\eta(B_j)^2 + f(A_rB_s)\eta(A_rB_s)^2}{f(A_i)\eta(A_i) + f(B_j)\eta(B_j) + f(A_rB_s)\eta(A_rB_s)}. \qquad [1]$$

It is easy to show that the normalized brightness of a mixture lies in between that of the dimmest and brightest species present.

The brightness of the protein complex A_i is $\varepsilon = i\cdot\varepsilon_M$, because it is composed of i labeled monomeric subunits. Consequently, the normalized brightness of A_i is $\eta(A_i) = i$ at wavelengths where CFP is excited. Similarly, the normalized brightness of B_j is $\eta(B_j) = j$. For conditions where the brightness of CFP and YFP are identical ($\varepsilon_M = \varepsilon_A = \varepsilon_B$), the normalized brightness of A_rB_s is $\eta(A_rB_s) = r + s$. But if only YFP is excited, then the normalized brightness of A_rB_s is $\eta(A_rB_s) = s$, whereas if CFP is exclusively excited, the brightness of the complex is $\eta(A_rB_s) = r$. Note that we choose experimental conditions where the influence of FRET on brightness is negligible (8). Thus, the normalized brightness encodes the stoichiometry of protein complexes. The evaluation of Eq. **1** requires a binding model that relates the individual protein species with one another.

A particular convenient situation for analyzing protein binding is given by the limiting case of stoichiometric binding conditions. It is experimentally realized for protein concentrations well above the dissociation coefficient describing the reaction. In the stoichiometric binding regime, it is straightforward to determine the population fractions from the protein expression ratio r_{AB}, which in turn determines the normalized brightness through Eq. **1**. Different binding models vary in their stoichiometric composition of the protein complexes and lead to predictions that can be compared with experiment.

Results

Fluorescence Lifetime. The lifetime of the donor RXRLBD-CFP was first determined in the absence of acceptor as 2.5 ns. Its lifetime was measured also for a large number of cells expressing RXRLBD-CFP and SRC1NID-YFP before and after adding the ligand 9-*cis*-retinoic acid (9cRA). In the absence of ligand, the fluorescence lifetime of the donor RXRLBD-CFP is 2.5 ns, which is unchanged from its original value in the absence of SRC1NID-YFP. Thus, FRET is not detected in the absence of ligand. In the presence of ligand, the fluorescence lifetime of the donor varied for different coexpression ratios between 2.5 and 2.3 ns. This corresponds to a change of $<10\%$ in the fluorescence lifetime due to FRET. The observation of FRET confirms protein–protein interactions between RXRLBD-CFP and SRC1NID-YFP in the presence of ligand but does not provide specific information about the protein complexes formed. We exploit this result and determine the protein coexpression ratio from cells unexposed to ligand. Because FRET is absent under these conditions, the ratio is directly related to the dual-color intensity ratio without requiring lifetime measurements. Once the protein ratio is determined, the ligand is added for further characterization of the cells.

Brightness Profile. The goal of measuring the brightness profile is to identify the best coexpression ratio for probing protein interactions. Ideally, the proteins coexpression ratio should match the stoichiometry of the protein complex, so that all proteins are able to participate in the formation of the protein oligomer. For example, if A and B interact with 2:1 stoichiometry (A_2B), then the optimal coexpression ratio for these proteins is 2:1. This expression ratio ensures that under stoichiometric binding conditions all proteins form heterotrimeric A_2B, which leads to a normalized brightness of 3. A 4:1 coexpression ratio, on the other hand, will result in an excess of protein A without an available binding partner B, whereas a 1:1 coexpression results in an excess of protein B. The normalized brightness is <3 for these conditions due to the presence of an excess population that is unable to form a heterotrimer. The apparent brightness reaches its maximum value when the coexpression ratio matches the stoichiometry ratio of the oligomer.

We apply this approach to cells coexpressing SRC1NID-YFP and the CFP-tagged LBD of RXR (RXRLBD-CFP). Stoichiometric binding conditions are realized if the protein concentrations are much larger than the dissociation coefficient K_d of the oligomer. Thus, we selectively measure the brightness of cells with protein concentrations of at least 10 μM. Fig. 2 shows the normalized brightness determined at an excitation wavelength of 902 nm as a function of the protein coexpression ratio, $N_{\mathrm{RXRLBD}}/N_{\mathrm{SRC1NID}}$. The normalized brightness of the complex is $\approx$3.5 for

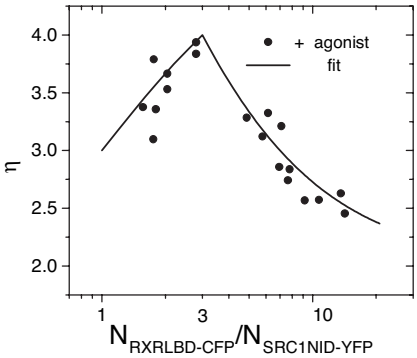

Fig. 2. Brightness profile of CV-1 cells expressing SRC1NID-YFP and RXRLBD-CFP. The normalized brightness is graphed as a function of the protein expression ratio, $N_{RXRLBD}/N_{SRC1NID}$. Cells with a total protein concentration of at least 10 μM were selected and excited at 902 nm. The solid line represents the predicted theoretical brightness for a 3:1 binding of RXRLBD-CFP and SRC1NID-YFP under stoichiometric binding conditions.

a 2:1 expression ratio, reaches a maximum of 4 at a 3:1 expression ratio, and slowly decreases with increasing expression ratio. The highest molecular brightness occurs at a 3:1 RXRLBD/SRC1NID protein coexpression ratio. This result suggests that the proteins form a SRC1NID(RXRLBD)$_3$ heterotetramer complex, provided that we are measuring under stoichiometric binding conditions.

Brightness Titration. To further characterize the interaction between both proteins, we studied the concentration dependence of their interaction. For this experiment, we needed cells with a 3:1 RXRLBD-CFP/SRC1NID-YFP expression ratio, because the brightness profile indicates that both proteins interact with this stoichiometry. To identify a sufficient number of cells, we accepted cells with expression ratios between 2.3 and 3.2. The brightness of each cell was measured at excitation wavelengths of 902 and 820 nm in the absence of the ligand 9cRA. After adding ligand, the brightness of the cells is remeasured at the same excitation wavelengths previously used. Fig. 3 graphs the normalized brightness of the cells as a function of protein concentration, which we refer to as brightness titration curves (7).

We first consider the normalized brightness in the absence of ligand for λ = 820 nm, which reflects the oligomeric state of RXRLBD-CFP, because this wavelength only excites the CFP chromophore. As a control RXRLBD-CFP was measured by itself. Its normalized brightness at low concentrations is 1, which indicates the presence of monomers, and reaches a value of $\approx$1.3 at high concentrations, which reflects the formation of some homocomplexes of RXRLBD-CFP (Fig. 3*A*). This result is in agreement with a previous characterization of RXRLBD-GFP (6, 7). Because RXRLBD is able to form homodimers, the brightness increase reflects the formation of a fraction of RXRLBD-CFP homodimers. Note that the brightness titration curve is unchanged upon adding SRC1NID-YFP. Thus, homodimer formation of RXRLBD is not influenced by the coactivator SRC1NID in the absence of ligand.

Next, we examine the brightness of RXRLBD-CFP in the presence of the agonist 9cRA at λ = 820 nm. RXRLBD-CFP is measured in the absence of SRC1NID as a control. Its normalized brightness increases from 1 to a value of 2 (Fig. 3*B*). This result agrees with a previous brightness measurement of RXRLBD-GFP and confirms the dimerization of RXRLBD at high concentrations in the presence of 9cRA. Adding the coactivator fragment SRC1NID in the presence of ligand leads to pronounced changes in the normalized brightness of RXRLBD-CFP. Its value starts at 1.5 and reaches 3 at high concentrations,

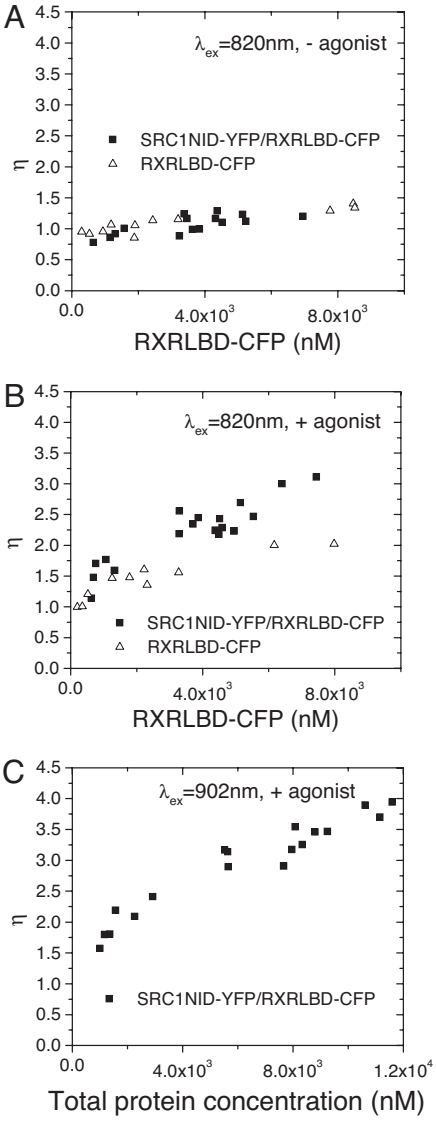

Fig. 3. Brightness titration curves taken in the nucleus of CV-1 cells. Cells expressing RXRLBD-CFP are measured as a control. The brightness values of cells coexpressing RXRLBD-CFP and SRC1NID-YFP with a ratio between 2.3 and 3.2 are measured. (*A*) Cells are measured at 820 nm in the absence of agonist. Normalized brightness of RXRLBD-CFP (triangles) and coexpressed with SRC1NID-YFP (squares) are shown as a function of protein concentration. (*B*) Cells are measured at 820 nm in the presence of 9cRA. Normalized brightness of RXRLBD-CFP (triangles) and coexpressed with SRC1NID-YFP (squares) are shown as a function of protein concentration. (*C*) Cells are measured at 902 nm in the presence of 9cRA. Normalized brightness of RXRLBD-CFP coexpressed with SRC1NID-YFP (squares) is shown as a function of protein concentration.

suggesting the formation of trimers of RXRLBD-CFP. This result is consistent with the formation of SRC1NID(RXRLBD)$_3$ heterotetramers as suggested from the brightness profile data. The graph in Fig. 3*B* demonstrates that the presence of SRC1NID promotes the formation of homocomplexes of RXRLBD, presumably through binding of RXRLBD to SRC1NID, although we cannot tell directly from the data presented because only RXRLBD-CFP is excited at 820 nm.

To address this point, we remeasure at λ = 902 nm, where CFP and YFP are coexcited and have the same brightness. Fig. 3*C* shows the brightness of cells coexpressing RXRLBD-CFP and SRC1NID-YFP in the presence of 9cRA. Oligomers are already present at low concentrations, because the normalized bright-

ness is >1. The brightness value grows with concentration and reaches a value of 4, which is consistent with the formation of tetramers. We deduced earlier from the data taken at 820 nm that RXRLBD-CFP exists as a trimer. Together these results imply a 3:1 stoichiometry for the protein complex of RXRLBD-CFP and SRC1NID-YFP in the presence of ligand, which agrees with the result obtained from the brightness profile (Fig. 2).

Thus, our data demonstrate that under stoichiometric binding conditions, a 3:1 complex of RXRLBD and SRC1NID is formed. Any excess of SRC1NID without a binding partner will be monomeric, whereas excess of RXRLBD will exist as a homodimer. For a quantitative comparison with the binding model described above, we calculated the normalized brightness with Eq. **1** as a function of the coexpression ratio assuming stoichiometric binding conditions. The theoretical curve shown as a solid line in Fig. 2 agrees with the measured brightness profile. Note that at protein ratios of <3 an excess of SRC1NID-YFP is present and reduces the brightness of the sample, whereas at ratios >3 an excess of $(RXRLBD\text{-}CFP)_2$ exists, which also leads to a reduction in the brightness.

We performed an additional experiment where the binding between RXRLBD and SRC1NID was investigated by labeling both proteins with GFP instead of using CFP and YFP. Although we cannot distinguish between both proteins anymore, a maximum brightness of 4 is expected for cells that express both proteins in a 3:1 ratio at high protein concentrations, which was confirmed by experiment (see supporting information (SI) Fig. 5). This experiment provides an additional control where hetero-FRET between CFP and YFP is absent and confirms the brightness analysis in the presence of FRET.

NID Fragment. The coactivator family, SRC1, SRC2, SRC3, contains the highly conserved short motif LXXLL, also termed NR box, which is responsible for interaction with NRs (11, 13). The NR interaction domain (NID) of the coactivator contains three NR boxes (Fig. 1). Our data agree with the binding of three RXRLBD molecules to the coactivator protein, which is suggestive of a role of each NR box in binding one RXRLBD molecule. To further investigate this point, we generated another truncation mutant (amino acid residues 570–737) that only contains two NR boxes (see Fig. 1) and labeled it as SRC1NIDT. We transfected cells with SRC1NIDT-YFP and RXRLBD-CFP and selectively measured the brightness of cells with an RXRLBD-CFP concentration >10 μM. Their normalized brightness measured for λ = 902 nm is graphed as a function of the coexpression ratio, $N_{RXRLBD}/N_{SRC1NIDT}$ (Fig. 4*A*).

If deletion of one of the three NR boxes reduces the number of recruited RXRLBD molecules by one, the maximum normalized brightness should be reduced to 3. Although the experimentally observed maximum is close to 3, it exceeds this value slightly. In addition, the brightness profile shows no sharp maximum at a 2:1 ($N_{RXRLBD\text{-}CFP}/N_{SRCNID\text{-}YFP}$) protein expression ratio as expected for a $(RXRLBD)_2$SRC1NIDT trimer. To explain the presence of brightness values that exceed the theoretical limit of our model requires the consideration of protein complexes larger than a trimer. One scenario to explain the data is the aggregation of the truncated coactivator, which would result in larger complexes. To check this hypothesis, we measured the brightness of the cells at 960 nm where only YFP is excited. The normalized brightness of SRC1NIDT-YFP is 1 at low and high coexpression ratios but is >1 for coexpression ratios close to one (Fig. 4*B*). Thus, a fraction of the SRC1NIDT-YFP molecules form homocomplexes, presumably dimers. The larger scatter in the data as compared with the data in Fig. 2 most likely reflects that strict stoichiometric binding conditions are not established yet. The fractional amount of complex depends in this case on the concentration, which leads to additional scatter of the brightness.

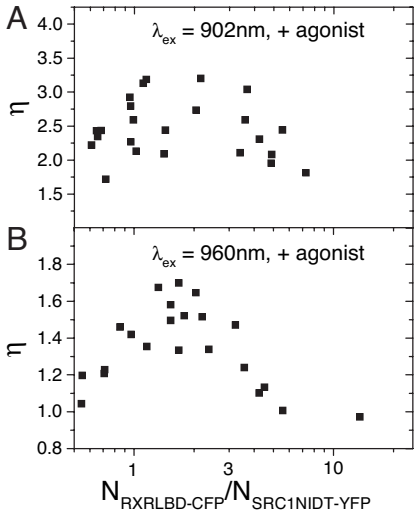

Fig. 4. Brightness profile of CV-1 cells expressing SRC1NIDT-YFP and RXRLBD-CFP. The normalized brightness is graphed as a function of the protein expression ratio, $N_{RXRLBD}/N_{SRC1NIDT}$. (*A*) Cells with concentrations of RXRLBD-CFP >10 μM were selected and excited at 902 nm. (*B*) Cells with concentrations of RXRLBD-CFP >10 μM were selected and excited at 960 nm.

Homoassociation of SRC1NIDT only appears when RXRLBD and SRC1NIDT have similar concentrations. The data point toward an RXRLBD dimer induced linkage between two SRC1NIDT molecules. In the presence of a large excess of RXRLBD, the chance that two SRC1NIDT recruit the same RXRLBD dimer is negligible, which explains the monomeric brightness of the coactivator fragment at large coexpression ratios. At low coexpression ratios, not enough RXRLBD is available to link the coactivator fragment together, resulting in a large excess of monomeric coactivator. Only at approximately equal concentrations of RXRLBD and SRC1NIDT are the conditions correct for a large fraction of linked SRC1NIDT.

Discussion

The focus of this paper is the introduction of a methodology with the power to quantify the stoichiometry of protein heterocomplexes in living cells. We chose the LBD of RXR and the NID of SRC-1 as a simple protein system to illustrate our strategy. Note that because we used domains instead of the full-length protein, we cannot provide direct information about the interactions of the biologically relevant full-length proteins. However, our results show a promising experimental approach with which to address such questions. The brightness profile identifies protein coexpression conditions that provide the brightest and therefore largest protein complexes. This experiment is followed up by a brightness titration at the coexpression ratio that yielded the brightest sample in the brightness profile measurement. These measurements provide information about the presence of stoichiometric binding conditions. Selective excitation of CFP and YFP, together with coexcitation of both fluorophores, determines the oligomeric composition of the protein complex formed.

The presence of stoichiometric binding conditions for a hypothetical A_rB_s complex at the highest measured concentrations is inferred as follows. The brightness profile has to reach a maximum for a coexpression ratio of r/s with a normalized brightness value of $r + s$ if both proteins are coexcited. This necessary condition is satisfied in our case for $r = 3$ and $s = 1$. In the next step, the concentration dependence of the brightness of cells with a coexpression ratio r/s is investigated. A brightness titration curve that reaches a plateau with a normalized bright-

ness value of $r + s$ indicates the presence of stoichiometric binding conditions. In our case the plateauing was not observed. The brightness value increased monotonically over the concentration range studied and only reaches the expected value for the highest concentrations (Fig. 3*C*). To check whether stoichiometric binding conditions are achieved at the highest measured concentration, we use another test. Stoichiometric binding dictates the presence of a pure A_rB_s species for a coexpression ratio of r/s. A pure species requires a normalized brightness of r if protein A is selectively excited. Under nonstoichiometric binding conditions, a mixture of species is present, which leads to normalized brightness values for A that differ from r. The same principle applies to the normalized brightness of protein B, which predicts a brightness of s for a pure species. Our data confirmed that the normalized brightness value of protein A is $r = 3$. The normalized brightness of protein B has to be $s = 1$, because brightness values are additive under our experimental conditions. Thus, we conclude that stoichiometric binding conditions are reached at a concentration of 10 μM.

The observation that three RXRLBD molecules bind to the coactivator is a surprising finding. Because NRs generally interact with their respective response element as dimers, it has generally been assumed that NRs bind to the coactivator as dimers by binding to two of the three available NR boxes. The reason for the presence of three NR boxes in the SRC coactivator is not understood. It is believed that this enables coactivators to bind to a large variety of NRs. This view is supported by studies of short peptides containing a single NR box reporting preferential binding of specific NR boxes to NRs (14–16). However, such studies neglect the influence of protein conformation on binding (17). We demonstrated that eliminating NR box III by truncating SRC1NID changes the binding properties in a nontrivial way. It resulted in NR-induced association of the truncated SRC1 fragment, which is absent in SRC1NID. This result underscores the importance of protein conformation in the interaction between the coactivator and its NR. Note that brightness analysis is currently the only technique capable of detecting such changes in the binding mechanism. This feature opens the possibility to directly characterize in cells the changes in molecular interactions as a result of protein modifications.

The experimental approach used here is very similar to performing biophysics in a test tube. We change the composition and concentration of the sample in a systematic manner and observe its response but use cells instead of test tubes. By performing a titration, we have to examine proteins at nonphysiological concentrations but gain useful information about the formation of protein complexes that ultimately are needed for a description of cellular events at the molecular level. For experimental ease, the presence of endogenous protein was neglected in this study. Endogenous protein competes with fluorescently tagged exogenous protein in forming oligomers. Consequently, the brightness of protein complexes that contain endogenous protein will be reduced, because such complexes contain nonfluorescent endogenous protein. We previously showed using Western blot analysis that endogenous RXR is absent in the cell line used for the experiments (7), but endogenous coactivator SRC-1 is present. The approach taken here is to exploit the wide concentration range of expressed protein accessible by transient transfection. Protein interactions are detected by FFS when the exogenous protein concentration is equal to or greater than the endogenous protein concentration, because the fluorescently labeled protein is starting to represent the dominant population. Therefore, the population of protein complexes containing more than one fluorescent protein increases leading to a normalized brightness that is >1. In other words, detecting the increase in the brightness as a function of protein concentration is not only proof of the existence of protein interactions but also provides an upper limit for the endogenous protein concentration present in the cell. If the exogenous concentration becomes high enough, the presence of the endogenous population becomes negligible. In this limit the measured brightness reflects the true oligomeric composition of the protein complex. If in addition the protein concentration exceeds the binding affinity, stoichiometric binding conditions are established, which allow an exact determination of the composition of the protein complex. The advantage of this approach is that it is straightforward to implement, but it requires working at concentrations that exceed the endogenous one. An alternative approach, which avoids this complication, is the use of siRNA technology to selectively knock down the endogenous population.

Although FRET is routinely used to detect protein–protein interactions in cells (18), it has a number of limitations. For example, the absence of a FRET signal cannot rule out the presence of protein association. In addition, changes in the distance and orientation of the chromophores within a protein complex, or partial dissociation of the complex, result in changes in the FRET efficiency that are hard to disentangle and quantify. Thus, FRET is generally not able to quantify the composition of a protein complex. In summary, a positive FRET result is an excellent marker that proteins interact, but without providing quantitative details, whereas a negative FRET result cannot rule out protein interactions.

Brightness analysis, on the other hand, provides considerably more detail about the protein complex than FRET. It readily identifies homointeractions and heterointeractions of proteins (7, 8) and determines their composition under stoichiometric binding conditions. An alternative and more powerful approach would be the direct resolution of all species present by brightness analysis. This approach would work at all concentrations but is a much more difficult task because it requires the resolution of at least three species from a single measurement, which has not been achieved yet. Thus, our method offers the only practical way at present to determine the stoichiometry of a higher-order heterocomplex.

The detection of protein interactions by brightness does not require FRET. Thus, brightness analysis is useful for characterizing protein complexes that have no FRET and works with pairs of fluorophores that are unsuitable for FRET measurements. In principle, the presence of FRET affects the brightness of the donor and acceptor. However, it is possible to find conditions where the brightness of a protein complex becomes independent of FRET. This situation is essentially realized when the loss of brightness by the donor is compensated by the increase in brightness of the acceptor. We have shown that this is valid on our instrument for the CFP/YFP pair at an excitation wavelength at 902 nm (8) and also holds true for excitation at 820 nm. Thus, judicious choice of instrumental parameters decouples brightness from FRET. The current experiments do not distinguish the protein species by the emission color but by changing the excitation wavelength. The emission spectra of CFP and YFP overlap significantly, which complicates dual-color FFS analysis considerably. It is far more sensitive and advantageous to selectively excite the protein species through changes in the excitation wavelength, whereas the fluorescence is collected irrespective of color.

Our experiments require knowledge of the protein coexpression ratio. It is determined by dual-color intensity ratio measurements. However, the presence of FRET leads to a bias. Thus, we routinely measure the lifetime of the donor to determine the apparent FRET efficiency and use it to correctly identify the coexpression ratio of the cell (8). In the present study, no FRET was detected for cells expressing SRC1NID and RXRLBD in the absence of 9cRA. We, therefore, are able to calculate the coexpression ratio directly from the measured dual-color intensity ratios.

In summary, brightness analysis by FFS is a biophysical technique that has great potential for significant contributions to our understanding of cell biology. Here we demonstrated that FFS is capable of identifying the formation of a protein heterotetramer in living cells. Note that this study presents a quantitative determination of a higher-order oligomer in living cells, which has not been reported previously. Fluorescent proteins such as CFP and YFP are used to determine the concentration and stoichiometry of the protein complex. Brightness analysis offers a number of advantages over FRET, extends our ability to investigate protein interactions in cells, and provides quantitative information that previously could be obtained only by *in vitro* biophysical analyses.

Materials and Methods

Experimental Setup. The beam of a mode-locked Ti:sapphire laser (Tsunami; Spectra Physics, Mountain View, CA) pumped by an intracavity doubled Nd:YVO_4 laser (Millenia; Spectra Physics) was directed into a modified Axiovert 200 microscope (Zeiss, Thornwood, NY) equipped with a 63X Plan Apochromat oil immersion objective (N.A. = 1.4) as described (8). The epifluorescence mode of the instrument was used to locate and position cells residing in a chamber slide (Nalge Nunc International, Rochester, NY) mounted on an electronic stage (MS-2000 XYZ; ASI, Eugene, OR). The location of each measurement within cells was recorded from the electronic readout of the *x-y* position of the stage. Once a cell was selected, the excitation source was switched to the Ti:sapphire laser for FFS experiments. The laser power measured after the objective was 1.2 and 0.6 mW for excitation wavelengths of 902 and 820 nm, respectively. We first determined for excitation at 902 nm the fluorescence intensity ratio of the emitted fluorescence by using a dichroic mirror with a center wavelength of 525 nm (525DCXRU; Chroma Technology, Brattleboro, VT) that split the fluorescence into two detection channels. The intensities of the dual-color experiment were recorded by two avalanche photo diodes (APD) (Model SPCM-AQR-14; PerkinElmer, Vaudreuil, Canada). The calculated intensity ratio provides information about the coexpression ratio of the CFP- and YFP-labeled proteins (8). For the second measurement, the fluorescence emission was redirected to another microscope port where the fluorescence intensity was recorded for brightness analysis with another APD. The data acquisition time for measuring the intensity ratio is ≈5 s, whereas brightness measurements require ≈30 s. The TTL-output of each APD unit was connected to a PCI data acquisition card (ISS, Champaign, IL), which stores the complete sequence of photon counts using a sampling frequency of 20 kHz. The recorded photon counts were analyzed with programs written for IDL 5.4 (Research Systems, Inc., Boulder, CO).

Additional Sample Preparation and Experimental Protocols. The construction of expression vectors, cell preparation, and brightness calibration are described in detail in *SI Text*. Also described is the experimental protocol of the fluorescence lifetime and protein coexpression ratio measurements.

This work was supported by National Institutes of Health Grant GM64589 and National Science Foundation Grant MCB-0110831.

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