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Organization of Higher-Order Oligomers of the Serotonin$_{1A}$ Receptor Explored by Homo-FRET in Live Cells

Sourav Ganguly, Andrew H. A. Clayton, and Amitabha Chattopadhyay

We first discuss the case of a dilute population of molecules tagged to a fluorophore in a given state of oligomerization using the theoretical framework developed earlier (1). We assume that all the molecules are fluorescently tagged and their fluorescence properties are uniform. Each oligomeric species is characterized by its anisotropy, *i.e.*, an oligomeric species with i monomers will exhibit an anisotropy of r_i (*e.g.*, r_1, r_2, r_3 and r_4 will be the anisotropy for pure monomer, dimer, trimer and tetramer, respectively).

The anisotropy of the monomer (r_1) is greater than the higher (N > 1) oligomeric forms due to depolarization of fluorescence emission by homo-FRET in case of oligomers. This gives rise to the condition:

$$r_1 > r_2 > r_3 > r_4$$

The anisotropy for an N-mer (an oligomer with N monomers) can be assumed to be r_1/N. Strictly speaking, this choice of anisotropy corresponds to the limit of efficient energy migration between randomly oriented yet rotationally fixed fluorophores. Although this condition may not be valid in the present case, r_i would still be a function of i.

The effect of photobleaching on the fluorescence anisotropy of such a population of homogeneous oligomers can be described as discussed below. For the case of a monomeric population, homo-FRET would be absent (by definition). Photobleaching of such a population therefore will not lead to any change in anisotropy. For a monomeric population, the anisotropy would be independent of the fractional photobleaching (denoted as x). In reality, it should be noted that photobleaching would lead to a loss of fluorescence intensity, and as $x \rightarrow 1$, signal-to-noise ratio could be a limiting factor. For a population of dimers, progressive photobleaching would lead to a gradual increase in anisotropy. For a pure population of dimers, the anisotropy of the dimer is given by r_2 ($r_2 < r_1$). Since each monomer contains one fluorophore, three types of dimers would be generated (differing in the fluorescent state of the attached fluorophore) upon progressive photobleaching. These are: R*—R*, R*—R, and R—R where R* and R represent fluorescent and photobleached states of the fluorophore. Of these, R—R would be

nonfluorescent (dark) and therefore not contribute to the measured anisotropy. Assuming photobleaching to be random, the R*—R* population of dimers would be predominantly bleached for small values of *x*. With increase in the extent of photobleaching (*i.e*., with increasing *x*), the fraction of R*—R would increase. This species (R*—R) would be incapable of homo-FRET (due to the loss of a partner for homo-FRET). The anisotropy of R*—R would be essentially same as the monomeric anisotropy (r_1). In such a scenario, the resultant anisotropy of the population would change from r_2 (initial) to eventually to r_1, at the limit of fractional photobleaching $x \rightarrow 1$. This can be expressed binomially as:

$$r(2) = r_1 x + r_2(1-x)$$

where *r(2)* is the resultant anisotropy of the population as a function of *x*. Similarly, for a pure population of trimers, one obtains:

$$r(3) = r_1 x^2 + 2r_2 x(1-x) + r_3(1-x)^2$$

where *r(3)* corresponds to the resultant anisotropy of a trimeric population. Likewise, in case of tetramers, the resultant anisotropy is given by:

$$r(4) = r_1 x^3 + 3r_2 x^2(1-x) + 3r_3 x(1-x)^2 + r_4(1-x)^3$$

In general, for a homogeneous population of N-mer, the resultant anisotropy is given by:

$$r(N) = A_1 r_1 x^{(N-1)}(1-x)^0 + A_2 r_2 x^{(N-2)}(1-x)^1 + A_3 r_3 x^{(N-3)}(1-x)^2 + ... + A_N r_N x^0 (1-x)^{(N-1)}$$ Thes

e expressions are obtained by noting that for a population of N-mer, one has a polynomial of order (N-1) with the coefficients in the expansion (A_1, A_2, . . . A_N) derived from the $(N-1)^{th}$ row of the Pascal's triangle.

In a cellular milieu (particularly in a microheterogeneous media such as the biological membrane), a more realistic scenario would be the possible coexistence of various oligomeric forms in the same population. In order to address the change in fluorescence anisotropy upon photobleaching in such a population, we consider a Poisson distribution of monomers with the mean number corresponding to the order of oligomerization of the majority species in the population. For this, we consider a distribution of oligomers with oligomeric states 1 to N, each

with a corresponding mole fraction χ_N. The resultant anisotropy as a function of x can be then be described as:

$$r(N,\chi_N) = \sum_N N\chi_N r(N,x) \Big/ \sum_N N\chi_N$$

Interpretation of anisotropy data after photobleaching

As discussed above, the anisotropy enhancement after photobleaching can be interpreted in terms of oligomer size. For a monomeric population, the anisotropy as a function of fluorophore labeling (f) is invariant to photobleaching:

$$r^{monomer}(f) = r_1$$

For a dimeric population, the predicted anisotropy is given by:

$$r^{dimer}(f) = (1\text{-}f)r_1 + fr_2$$

where r_1 is the anisotropy of a singly-labeled dimer (R*—R; partially photobleached, no homo-FRET) and r_2 is the anisotropy of a doubly-labeled dimer (R*—R*). For a tetrameric population,

$$r^{tetramer}(f) = (1\text{-}f)^3 r_1 + 3f(1\text{-}f)^2 r_2 + 3f^2(1\text{-}f)\, r_3 + f^3 r_4$$

In the context of a mixed monomer-dimer-tetramer population, the total anisotropy as a function of labeling is given by the equation:

$$r^{total}(f) = a\, r(f)^{monomer} + b\, r^{dimer}(f) + (1\text{-}a\text{-}b)\, r^{tetramer}(f)$$

where a, b and (1-a-b) correspond to the monomer, dimer and tetramer fractions, respectively. In principle, fitting anisotropy enhancement after photobleaching to these equations should yield the required fractions. However, this analysis requires bleaching to completion, which is not possible in a realistic situation due to loss of intensity (as mentioned above) and possible cytotoxic effects. Instead, the initial anisotropy and the linearly extrapolated anisotropy can be used.

When all molecules are labeled, *i.e.*, $f = 1$,

$$r^{total} = a\, r^{monomer} + b\, r^{dimer} + (1\text{-}a\text{-}b)\, r^{tetramer}$$

The linearly extrapolated anisotropy, *i.e.*, apparent $f = 0$, is obtained by extrapolating the tangent to the function close to $f = 1$. The equations describing monomer, dimer and tetramer can be described as:

$$d(r(f)^{monomer})/df = 0;\ r(f = 0) = r_1$$

$$d(r(f)^{dimer})/df = r_2 - r_1;\ r(f = 0) = r_1$$

$$d(r(f)^{tetramer})/df = 3(r_4 - r_3);\ r(f = 0) = 3r_3 - 2r_4$$

It should be noted that the extrapolated anisotropies of monomers and dimers are equal to the monomeric anisotropy (only due to rotation), but the extrapolated anisotropy of tetramers (or other oligomers) is less than the monomeric anisotropy, provided $r_2 > r_4$. This results from the fact that anisotropy *vs*. labeling (photobleaching) curve for oligomers exhibits upward curvature. Using the linearly extrapolated anisotropy ($f = 0$),

$$r^{total}\ (f \rightarrow 0) = (a + b)\ r_1 + (1\text{-}a\text{-}b)(\ 3r_3 - 2r_4)$$

It follows that the fraction of oligomeric (in this example tetrameric) forms can be determined from the extrapolated anisotropy after photobleaching using the above equation. It is apparent from this equation that *the difference between the monomeric anisotropy and the extrapolated anisotropy is directly proportional to the fraction of oligomers*. This approach is particularly useful when comparing a series of measurements where a qualitative indication of relative amounts of oligomers is needed. For example, Bader et al. (2) estimated values for the relevant anisotropies for eGFP-tagged monomers, dimers and oligomers, to be: $r^{monomer} = 0.38$, $r^{dimer} = 0.31$, $r^{oligomer} = 0.276$.

Reference

1. Yeow EKL, Clayton AHA (2007) Enumeration of oligomerization states of membrane proteins in living cells by homo-FRET spectroscopy and microscopy: theory and application. *Biophys. J.* 92:3098-3104.

2. Bader AN, Hofman EG, Voortman J, en Henegouwen PM, Gerritsen HC (2009) Homo-FRET imaging enables quantification of protein cluster sizes with subcellular resolution. *Biophys. J.* 97:2613-2622.