Oligomer Size of the Serotonin 5-HT_{2C} Receptor revealed by
Fluorescence Correlation Spectroscopy with Photon Counting Histogram Analysis:
evidence for homodimers without monomers or tetramers.

Katharine Herrick-Davis\(^1\), Ellinor Grinde\(^1\), Tara Lindsley\(^1\),
Ann Cowan\(^2\), and Joseph E. Mazurkiewicz\(^1\).

\(^1\)Center for Neuropharmacology & Neuroscience, Albany Medical College, Albany, NY 12208
\(^2\)Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT 06030

*Running title: FCS analysis of GPCR oligomer size

To whom correspondence should be addressed: Katharine Herrick-Davis, Center for Neuropharmacology & Neuroscience, MC-136, Albany Medical College, 47 New Scotland Avenue, Albany, NY 12208, Tel.: (518) 262-6357; Fax: (518) 262-5799; E-mail: daviskh@mail.amc.edu

Keywords: GPCR, homodimer, FCS, PCH, 5-HT\(_{2C}\)

**Background:** The functional signaling unit of G protein coupled receptors is debated to be a monomer, dimer or higher order oligomer.

**Results:** Fluorescence correlation spectroscopy and photon counting histogram analysis, with single molecule sensitivity, identified 5-HT\(_{2C}\) receptor dimers without monomers or tetramers.

**Conclusion:** Dimers are the basic signaling unit.

**Significance:** Bivalent ligands may have therapeutic potential.

**Summary**

Fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) are techniques with single molecule sensitivity that are well suited for examining the biophysical properties of protein complexes in living cells. In the present study, FCS and PCH were applied to determine the diffusion coefficient and oligomeric size of G-protein-coupled receptors (GPCR). FCS was used to record fluctuations in fluorescence intensity arising from fluorescent-tagged 5-HT\(_{2C}\) receptors diffusing within the plasma membrane of HEK293 cells and rat hippocampal neurons. Autocorrelation analysis yielded diffusion coefficients ranging from 0.8\(\mu\)m\(^2\)/s to 1.2\(\mu\)m\(^2\)/s for fluorescent-tagged receptors. Since the molecular brightness of a fluorescent protein is directly proportional to the number of fluorescent proteins traveling together within a protein complex, it can be used to determine the oligomeric size of the protein complex. FCS and PCH analysis of fluorescent-tagged 5-HT\(_{2C}\) receptors provided molecular brightness values that were twice that of GFP and YFP monomeric controls, similar to a dimeric GFP control, and unaltered by 5-HT. Bimolecular fluorescence complementation of the N- and C-terminal halves of YFP attached to 5-HT\(_{2C}\) receptors was observed in ER/Golgi and plasma membranes with a brightness equal to monomeric YFP. When GFP-tagged 5-HT\(_{2C}\) receptors were co-expressed with a large excess of untagged, non-fluorescent 5-HT\(_{2C}\) receptors, the molecular brightness was reduced by half. PCH analysis of the FCS data was best described by a one component dimer model without monomers or tetramers. Therefore, it is concluded that dimers represent the basic signaling unit.

Approximately 4% of the human genome encodes G-protein-coupled receptors (GPCR), one of the largest families of plasma membrane signaling proteins in the human body. These receptors have been successful targets for drug development, with a significant fraction of currently prescribed medications targeting GPCR. While GPCR were discovered decades ago, there is still great debate as to what constitutes the functional signaling unit in vivo: is it a monomer, dimer, or higher order oligomer? Several studies have reported that monomeric GPCR can be functional with respect to G protein activation (1,2) and that GPCR monomers can be detected in
recombinant cells (3-5). However, GPCR dimers/oligomers have been identified in native tissues and primary cultures (6-8) and it has been reported that G protein activation is maximal for the dimeric form of 5-HT receptors (8-10). In addition, dimerization has been reported to be an essential step in proper protein folding for exit from the endoplasmic reticulum (11-13). For class A GPCR, homo- and hetero-dimerization have been reported to regulate ligand binding, second messenger activation and receptor trafficking, and agonists have been reported to increase, decrease or have no effect on GPCR dimerization (reviewed in 14). In the case of class C GABA\textsubscript{B} receptors, the functional significance of dimerization is clear. Heterodimerization between GABA\textsubscript{B}R1 and GABA\textsubscript{B}R2 receptors in the ER is essential for trafficking and expression of functional heterodimers on the plasma membrane (15-17).

Currently, there is no consensus as to the oligomeric size of class A GPCR. Studies of class A GPCR have reported dimers (8,18-20), tetramers (21,22), and higher order oligomers (13,23). The most commonly employed methods include co-immunoprecipitation (co-IP), resonance energy transfer (RET), and bimolecular fluorescence complementation (BiFC). While co-IP provides information about direct protein-protein interactions, it requires solubilization of the proteins and thus can’t be applied to live cells. RET and BiFC can be applied to living cells, but they provide an indirect measure of protein-protein interaction by measuring the proximity of fluorescent probes attached to the parent proteins. More recently, single particle tracking methods such as total internal reflection fluorescence imaging (TIRF) have been employed. Studies using TIRF imaging have suggested that M\textsubscript{1}-muscarinic receptors (24) and N-formyl peptide receptors (25) may exist in a monomer-dimer equilibrium on the plasma membrane. While these studies represent a significant advance in terms of understanding receptor distribution and dynamics in plasma membranes, TIRF imaging has a limit of resolution of 200nm and lacks single molecule sensitivity for proteins on the order of 5nm, such as GPCR.

Fluorescence correlation spectroscopy (FCS) provides a good alternative for investigating the diffusion and oligomerization of plasma membrane proteins. It requires very low protein expression levels, making it suitable for studying receptors at physiological expression levels, and it has single molecule sensitivity. FCS records the fluctuations in fluorescence intensity arising from individual fluorescent molecules, in a temporal manner (26,27). Combining confocal microscopy with FCS has led to the development of sensitive methods for monitoring protein dynamics in living cells (28-34). Confocal microscopy-based FCS experiments are performed by focusing a laser beam into a small diffraction-limited spot (0.3\textmu m) using a high numerical aperture objective to obtain a detection or observation volume on the order of 10\textsuperscript{-15} liters. As individual fluorescent molecules pass through the observation volume, and are excited by a laser, the emitted fluorescence is recorded by a photon-counting detector. The autocorrelation of the fluorescence signal depicts the fluctuations in fluorescence intensity as a function of particle number and diffusion time. FCS has been used to monitor diffusion and ligand binding for ion channels, tyrosine kinase receptors and GPCR (reviewed in 35). Fluorescence cross-correlation spectroscopy, which compares the autocorrelation signals from two different fluorescent probes, has been applied to study homo- and hetero-dimers of somatostatin receptors (36), epidermal growth factor receptors (37), ciliary neurotrophic factor receptors (38), and estrogen receptors (39).

Information about the oligomeric size of a protein cluster can be obtained by analyzing the amplitude of the fluctuations in fluorescence intensity measured in an FCS experiment. A photon counting histogram (PCH) can be generated from the FCS data and used to determine the molecular brightness of a given fluorescent species or a mixture of fluorescent species of differing molecular brightness. PCH theory was developed and applied as a confocal microscopy-based method to measure the number of photon counts produced by individual fluorescent molecules in a small, laser-illuminated observation volume (40). The term “molecular brightness” is used to describe the number of photon counts per molecule. Since the molecular brightness of a protein oligomer is directly proportional to the number of fluorescent molecules within the protein complex, it can be used to determine the oligomeric size of a protein complex. Molecular brightness analysis has been used to explore the oligomeric state of nuclear retinoid X receptors.
FCS analysis of GPCR oligomer size

In the present study, the serotonin 5-HT_2C receptor was chosen as a model class A GPCR to investigate receptor diffusion dynamics and oligomer size using FCS and PCH. 5-HT_2C receptors are widely expressed throughout the brain in regions including the choroid plexus, hippocampus, hypothalamus, striatum and cortex (45,46). They have been proposed to play a role in appetite, mood, sleep, endocrine regulation, and drug addiction (46-48). Many different classes of psychoactive agents interact with 5-HT_2C receptors including hallucinogens, antipsychotics, antidepressants, and anxiolytics. Thus the 5-HT_2C receptor has been targeted for drug development for obesity, anxiety, depression, and schizophrenia (48). RET and cysteine cross-linking studies are consistent with the hypothesis that 5-HT_2C receptors form homodimers in recombinant cells (9,11,49).

Experimental Procedures

Plasmid DNA constructs - cDNA encoding the 5-HT_2C receptor (VSV isoform) was cloned into the pEGFP, pEYFP and mCherry vectors (Clonetech) at EcoRI/BamHI to create chimeric receptors with fluorescent tags on the C-terminus of the 5-HT_2C receptor. A tandem GFP construct was made by PCR of the GFP open reading frame and subsequently cloned back into the pEGFP plasmid. Site-directed mutagenesis (Stratagene) was used to create an A206K mutation in all GFP constructs to eliminate potential aggregation (50). BiFC pairs (N-YFP and C-YFP) were made by site-directed mutagenesis using the 5-HT_2C/YFP cDNA as the starting template. 5-HT_2C/N-YFP was made by inserting a BamHI site at amino acid 156 of the YFP, followed by BamHI digest to remove amino acids 1-155 of the YFP, and subsequent re-ligation. Beta-arrestin2 was a generous gift of the Lefkowitz laboratory, and was cloned into pECFP plasmid to create the beta-arrestin/CFP construct used in the beta-arresting recruitment assay.

Cell culture and transfection - HEK293 cells (ATCC) were cultured in Dulbecco’s Minimal Essential Medium (cellgro) with 10% FBS (HyClone) in a humidified chamber at 37°C, 5% CO_2. HEK293 cells were plated in 6 well plates fitted with 25mm poly-D-lysine coated glass coverslips (Fisher) at a density of 7 x 10^5 cells per coverslip and transfected with 30ng of the indicated plasmid DNA using lipofectamine reagent (Invitrogen) for five hours. Following transfection, cells were cultured in DMEM (without phenol red) with 10% charcoal stripped serum (Gibco) for 20 hours at 37°C, 5% CO_2.

This transfection protocol results in 5-HT_2C receptor expression levels of 1.1+/-.0.2 pmol/mg protein, determined by radioligand binding using a K_d concentration (1nM) of ^3H-mesulergine measured as previously described (9). For a typical transfection efficiency of 30%, this yields an approximate 5-HT_2C receptor expression level of 6 pmol/mg protein for the transfected HEK293 cells used in the FCS studies. Previous studies have determined endogenous 5-HT_2C receptor expression levels in choroid plexus epithelial cells to be in the range of 5-10 pmol/mg protein (51).

Neuronal cultures were prepared from hippocampi of fetal Sprague-Dawley rats (Taconic Farms) at gestational day 19 as previously described (52). Briefly, hippocampi were dissected and dissociated in HEPES-buffered salt solution with trypsin (0.25% for 15 min at 37°C) and triturated with a fire-polished pipette. Dissociated neurons were washed in HEPES-buffered salt solution and were transfected with plasmid containing 5-HT_2C/YFP cDNA using the Nucleofector kit from Amaxa according to the manufacturer’s instructions. Following transfection, neurons were plated in MEM with 10% heat-inactivated horse serum at a density of 6,000 cells/cm^2 on glass coverslips pre-coated with poly-D-lysine. Neurons were allowed to adhere to coverslips for 3 h, then the coverslips were placed...
in 60mm dishes of confluent astrocytes in serum-free neuron maintenance medium (MEM with N2 supplement, 0.1 mM pyruvate, 10 mM HEPES, conditioned by exposure to confluent cortical astrocytes for two days), and positioned so that they faced, but did not contact the astrocytes. Twenty-four hours post-transfection, coverslips with plated neurons were washed twice with HEPES-buffered MEM (without phenol red) and placed in a viewing chamber with 1ml of HEPES-buffered MEM (without phenol red) in preparation for the FCS experiments.

For 5-HT treatment, 5-HT was diluted in HEPES-buffered MEM (without phenol red) and was added directly to the viewing chamber to a final concentration of 50nM. FCS measurements were initiated one minute following the addition of 5-HT.

Fluorescence correlation spectroscopy (FCS) - For FCS measurements, cells were washed twice with HEPES-buffered MEM (without phenol red) and the coverslip was placed in a viewing chamber with 1ml of HEPES-buffered MEM (without phenol red). FCS measurements were made using a Zeiss LSM-510 confocal microscope equipped with a ConfoCor3 unit (Carl Zeiss, Germany) at the Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT. One-photon excitation with a continuous argon ion laser was performed using a 40x (N.A. 1.2) C-apochromat water immersion objective to create an observation volume on the order of 0.5 femtoliters. Since the observation volume is not illuminated homogenously, optimal positioning of the plasma membrane within the center of the observation volume is critical for accurate determination of the molecular brightness of fluorescent-tagged membrane proteins. FCS measurements were made on the apical plasma membrane (directly above the cell nucleus) of HEK293 cells, or hippocampal neuronal cell body, transfected with fluorescent-tagged 5-HT2c receptors. Positioning of the plasma membrane in the center of the observation volume was achieved by scanning the sample along the Z axis while simultaneously monitoring the photon count rate. The Z position corresponding to the maximal photon counts per molecule was selected. FCS measurements were recorded at 23°C in HEPES-buffered MEM (without phenol red) for 100 seconds, as 10 consecutive 10 second intervals. As fluorescent-tagged receptors enter and diffuse through the observation volume they are excited by the laser. GFP, YFP and mCherry were excited at 488nm, 514nm, and 563nm, respectively, with a laser intensity of 0.15mW. The time-dependent fluctuations in fluorescence intensity were recorded on an avalanche photodiode as follows: emitted fluorescence that was captured by the objective was passed through a dichroic mirror, appropriate band pass filter, and was focused onto the avalanche photodiode using a pinhole of one airy unit (72µm). The recordings were analyzed by a digital temporal correlator (using non-linear least-squares minimization, Zeiss Aim 4.2 software) to calculate the autocorrelation function $G(t)$, which represents the time dependent decay in fluorescence fluctuation intensity as in equation 1,

$$G(\tau) = \frac{\langle \delta F(t) \cdot \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}$$

where $G(\tau)$ is the <time average> of the change in fluorescence fluctuation intensity ($\delta F$) at some time point $(t)$ and at a time interval later $(t + \tau)$, divided by the square of the average fluorescence intensity. Autocorrelation analyses were performed using the Zeiss Aim 4.2 software package with an autocorrelation bin time of 0.2µs. The resulting autocorrelation curve depicts the fluorescence intensity fluctuations as a function of particle number and diffusion time. The average dwell time of the fluorescent species within the observation volume $(\tau_D)$ is calculated from the mid-point of the autocorrelation curve. The diffusion coefficient $(D)$ for fluorescent-tagged plasma membrane proteins can then be calculated as in equation 2, where $\omega_r$ is the radial waist of the observation volume (0.28µm).

$$D = \frac{\omega_r^2}{4\tau_D}$$

The amplitude of the autocorrelation function at time zero, $G(0)$, is inversely related to the number of molecules $(N_{PSF})$ and calculated as in equation 3,

$$N_{PSF} = \frac{1}{G(0)-1} \cdot \gamma$$
where $\gamma$ is the point spread function (PSF) describing the shape of the observation volume. For one-photon excitation the observation volume is best described by a 3D Gaussian model where $\gamma = 0.35$. Topography studies have provided clear and convincing images demonstrating the 3D nature of plasma membranes (53). Thus a valid case can be made for choosing a 3D model for determining particle number and brightness. The average fluorescence intensity or average count rate ($k$) measured for a given sample depends on the number of fluorescent molecules ($N_{PSF}$) and their molecular brightness ($\varepsilon$), as described in equation 4.

Equation 4: $k = N_{PSF} \cdot \varepsilon$

Thus dividing the count rate ($k$) by the number of molecules ($N_{PSF}$) provides an estimate of the molecular brightness ($\varepsilon$) of the sample.

As controls, pEYFP and pEGFP plasmids were expressed in the cytosol of transfected HEK293 cells to estimate the molecular brightness of monomeric forms of YFP and GFP (with an A206K mutation to eliminate dimer formation, 50). A tandem construct (GFP-GFP with A206K mutations) was used to determine the molecular brightness of a dimer. For cytosolic YFP and GFP, FCS measurements were made in the cytoplasm near the nuclear envelope in the mid portion of the cell, to avoid sample thickness-dependent bias in brightness (54). A dilute solution (5nM) of purified monomeric GFP was used for comparison with cytosolic GFP to evaluate the contribution of background auto-fluorescence from cytoplasmic proteins. In addition, two plasma membrane controls were used: a 5-HT$_{2C}$/YFP BiFC pair and co-expression of 5-HT$_{2C}$/GFP with a large excess of untagged, non-fluorescent 5-HT$_{2C}$ receptor.

**Photon Counting Histogram (PCH)** - Fluorescence fluctuation data recorded during an FCS experiment can be used to generate photon counting histograms, which provide quantitative information about the number of fluorescent molecules and the number of photon counts per molecule (40). In the present study, cells transfected with fluorescent-tagged 5-HT$_{2C}$ receptors were selected with an average plasma membrane photon count rate ranging from 50kHz to 150kHz. Membrane regions containing ruffles, filopodia, and high concentrations of fluorescent proteins (>200kHz) were avoided. Ten measurements were made on the upper plasma membrane of each cell by monitoring the photon count rate for 100 seconds, as 10 consecutive 10 second observation periods. While the laser intensity was set to 0.15mW to minimize photo-bleaching, some photo-bleaching was apparent during the first 10 second observation period. Molecular brightness values were determined for each 10 second observation period. The average molecular brightness from the second through tenth observation periods was then calculated and reported as the molecular brightness for that cell. Segments of the fluorescence intensity trace that showed large spikes or drifts in fluorescence intensity (due to cell movement) were excluded from the analysis. To generate a histogram, each 10 second observation period was broken down into one million intervals or bins (PCH bin time = 10µs). Histograms were constructed (using the PCH module in the Zeiss Aim 4.2 software) in which the number of 10µs bins was plotted on the y-axis and photon counts on the x-axis. The resulting histogram depicts the number of bins that registered 1, 2, 3 photon counts etc. during one 10 second observation period. Since a constant intensity light source produces a photon count distribution that follows Poisson statistics, as fluorescent molecules enter and diffuse through the non-homogenously illuminated observation volume the fluctuations in fluorescence intensity result in a broadening of the Poisson distribution. This super-Poisson characteristic is observed in the tail of PCH curve. The data were fit to a one component model where concentration and molecular brightness were allowed to be free, and the first order correction was fixed at zero. Reduced chi square analysis was used to determine the goodness of fit.

**RESULTS**

Bimolecular fluorescence complementation (BiFC) with fluorescence correlation spectroscopy (FCS) and photon counting histogram (PCH) analysis were applied to study the diffusion dynamics and oligomeric size of 5-HT$_{2C}$ receptors in HEK293 cells and primary hippocampal neurons. Previously, we have shown that 5-HT$_{2C}$ receptors with C-terminal fluorescent tags exhibit normal trafficking and signaling properties in
HEK293 cells (55). Reported EC_{50} values for 5-HT-stimulated inositol phosphate production were 2.4+/−0.5nM and 2.8+/−0.9nM for fluorescent-tagged and un-tagged receptors, respectively (55). Our previous studies using resonance energy transfer are consistent with the hypothesis that 5-HT_{2C} receptors form homodimers (9,11). In the present study, these results were confirmed using BiFC (figure 1). Plasma membrane YFP fluorescence was observed in HEK293 cells 20 hours after co-transfection with 5-HT_{2C} receptor cDNAs labeled with the N- and C-terminal halves of YFP (5-HT_{2C}/N-YFP + 5-HT_{2C}/C-YFP) as shown in figure 1a. Complementation of YFP fluorescence is lower following co-expression of 5-HT_{2C}/N-YFP with β_{2}-adrenergic/C-YFP (figure 1b), but is similar in cells co-expressing β_{2}-adrenergic/N-YFP and β_{2}-adrenergic/C-YFP (figure 1c). When cells co-transfected with 5-HT_{2C}/N-YFP and 5-HT_{2C}/C-YFP were examined 16 hours post-transfection, YFP fluorescence was observed in the endoplasmic reticulum (ER) and Golgi apparatus (figure 1d).

In transfected primary hippocampal neurons 5-HT_{2C}/YFP receptors are targeted to the plasma membrane of the cell body and along the axonal and dendritic membranes (figure 2). Endogenous 5-HT_{2C} receptor expression in the hippocampus (occurring predominantly in CA3 and dentate gyrus) is low at post-natal day 3 (15% of maximal expression levels), but increases steadily over the subsequent three weeks of development (56). The cultures used in our study were isolated from fetal rats at gestational day 19 and were grown in culture for one day prior to the FCS experiments. Thus it is unlikely that the neurons were expressing endogenous 5-HT_{2C} receptors at the time of the FCS experiments.

To determine the diffusion coefficient and oligomeric size of 5-HT_{2C} receptors, FCS measurements were made on the upper plasma membrane of transfected hippocampal neurons (figure 2 insert) and HEK293 cells. When fluorescent-tagged 5-HT_{2C} receptors, freely diffusing within the plasma membrane, enter the observation volume they are excited by the laser and the resulting fluctuations in fluorescence intensity are recorded in real time (figure 3a). Autocorrelation analyses of the fluorescence fluctuations were performed using a non-linear least-squares fitting routine which graphically represents the autocorrelation function G(t) on the ordinate and diffusion time on the abscissa (figure 3b). The diffusion of fluorescent 5-HT_{2C} receptors within the plasma membrane is reported as the average dwell time (τ_{D}) of fluorescent 5-HT_{2C} receptors within the observation volume and is calculated from the mid-point of the autocorrelation decay curve. The biphasic autocorrelation curves shown in figure 3b are best fit by a two component model with a very fast component characteristic of the photo-physical properties of the fluorescent probe (τ_{D1}) and a slower component representing the translational diffusion of the 5-HT_{2C} receptor within the plasma membrane (τ_{D2}). For example, in HEK293 cells the diffusion for 5-HT_{2C} receptors tagged with GFP or mCherry (τ_{D2}) is the same even though the two probes have different photo-physical properties and thus have different τ_{D1} values. Residuals of the curve fit are shown in figure 3c.

Diffusion coefficients obtained for fluorescent-tagged 5-HT_{2C} receptors in hippocampal neurons and HEK293 cells are presented in table 1. All diffusion data best fit a two-component model with τ_{D1} values for photo-physical properties ranging from 50µs-60µs for YFP and mCherry to 230µs for GFP, and τ_{D2} values ranging from 17ms for 5-HT_{2C}/YFP and the 5-HT_{2C}/YFP BiFC pair to 25ms for 5-HT_{2C}/GFP and 26ms for 5-HT_{2C}/mCherry. The results for YFP-tagged 5-HT_{2C} receptors are similar to literature values for YFP-tagged adenosine-A1 (18ms) and bradykinin-BK2 (16ms) receptors in CHO cells and HEK293 cells, respectively (57, 58). Since the diffusion rate is dependent on how long the fluorescent molecules are detected within the observation volume, the observed diffusion rate will be influenced by the photo-physical characteristics of the fluorescent probe used to label the protein of interest. Different fluorescent probes have different photo-physical properties, different rates of blinking on and off, different photo-stability, quantum yield and laser intensity for excitation, all of which will play a role in determining the apparent diffusion rate of the labeled protein (35).

Interestingly, the diffusion coefficient for 5-HT_{2C}/YFP was slightly faster in hippocampal neurons than in HEK293 cells, suggestive of a more dynamic membrane environment in the hippocampal neurons. It is possible that there are
differences between the cell types in terms of the cargo or proteins associated with 5-HT\textsubscript{2C}/YFP. Also, neuronal membranes are highly specialized in terms of being able to transport proteins long distances along axons and dendrites, properties not shared by HEK293 cells. Diffusion rates of sodium channels in neurons have been shown be faster along the axon than at the axon initial segment (boundary between axon and soma) demonstrating heterogeneity of protein diffusion even within a neuron (59). Thus it is not surprising that the diffusion rate of 5-HT\textsubscript{2C} receptors differed between HEK293 cells and hippocampal neurons.

The molecular brightness of a fluorescent-tagged protein is determined from an FCS experiment by calculating the number of fluorescent molecules present in the observation volume (as in methods, equation 3) and then dividing the average count rate obtained from the fluorescence intensity trace by the number of fluorescent molecules. This analysis yields an estimate of the molecular brightness expressed as counts per second per molecule (CPSM). Molecular brightness values were determined for YFP- and GFP-tagged 5-HT\textsubscript{2C} receptors freely diffusing within the plasma membrane of hippocampal neurons and HEK293 cells using both FCS and PCH (table 2). The molecular brightness of 5-HT\textsubscript{2C}/YFP in hippocampal neurons was 17,286\textpm{}1,257 CPSM, similar to 5-HT\textsubscript{2C}/YFP expressed in HEK293 cells (17,763\textpm{}1057 CPSM). The molecular brightness of the 5-HT\textsubscript{2C}/YFP BiFC pair (9,466\textpm{}1791 CPSM) was roughly half that observed for 5HT\textsubscript{2C}/YFP and was similar to monomeric YFP (9,403\textpm{}349 CPSM). For 5-HT\textsubscript{2C}/GFP, the molecular brightness was 17,751\textpm{}426 CPSM, which was similar to tandem GFP (19,294\textpm{}703 CPSM). Interestingly, the molecular brightness of 5-HT\textsubscript{2C}/GFP was reduced by half when co-expressed with a three-fold excess of untagged 5-HT\textsubscript{2C} receptors (9,003\textpm{}314 CPSM) and was similar to monomeric GFP (9,266\textpm{}211 CPSM).

To determine the contribution of background auto-fluorescence from cytoplasmic proteins, a dilute solution (5nM) of purified monomeric GFP was evaluated. The molecular brightness of GFP in solution (8,920\textpm{}114 CPSM) was similar to GFP expressed in the cytosol of HEK293 cells (9,266\textpm{}211 CPSM), indicating that background auto-fluorescence from cytoplasmic proteins was minimal (approximately 4%) in our experimental set-up.

PCH examines the amplitude of the fluctuations in fluorescence intensity recorded during an FCS experiment to provide an estimate of the average molecular brightness of all fluorescent species present in the observation volume. PCH can be used to determine the molecular brightness of a given fluorescent species or a mixture of fluorescent species, and as such is a valuable tool for measuring protein oligomerization (40, 60). Figure 4 shows the histograms for plasma membrane GFP-tagged 5-HT\textsubscript{2C} receptors, as well as the tandem GFP control. For comparison, two cells with similar average count rates (110kHz-120kHz) were selected. The shape of the PCH is a function of the number of fluorescent molecules and their molecular brightness, and was the same for the tandem GFP and 5-HT\textsubscript{2C}/GFP (figure 4). The PCH results for GFP- and YFP-tagged receptors and controls are shown in table 2. The PCH results were the same as the results obtained with FCS analysis. Again, the molecular brightness of fluorescent-tagged 5-HT\textsubscript{2C} receptors was similar to the tandem GFP control, twice that of monomeric forms of GFP and YFP, twice that of the BiFC pair, and was reduced by half following co-expression with an excess of untagged 5-HT\textsubscript{2C} receptors. Reduced chi square values for fitting the data to a one-component PCH model were close to unity.

To determine the effect of 5-HT treatment on receptor diffusion rate and on molecular brightness, transfected cells expressing 5-HT\textsubscript{2C}/GFP were treated with 50nM 5-HT for one minute prior to FCS recording. There was no effect of 5-HT treatment on the plasma membrane diffusion rate of 5-HT\textsubscript{2C}/GFP and no change in molecular brightness of 5-HT\textsubscript{2C}/GFP following treatment with 5-HT (table 2). These results are consistent with our previous observation that agonist and inverse agonist treatment has no effect on FRET efficiency (61).

To ensure that 5-HT\textsubscript{2C} receptors were activated in our experimental set-up, HEK293 cells co-expressing beta-arrestin2/CFP and 5-HT\textsubscript{2C}/YFP were challenged with 5-HT. Beta-arrestin recruitment to the plasma membrane occurred within 30 seconds following application of 50nM 5-HT (figure 5), demonstrating that 5-HT\textsubscript{2C} receptors are activated in response to 50nM 5-HT.
FCS analysis of GPCR oligomer size

DISCUSSION

FCS measures fluctuations in fluorescence intensity from individual fluorescent molecules, or in this case fluorescent-tagged 5-HT_{2C} receptors diffusing with the plasma membrane. This is accomplished by focusing a laser through a high numerical aperture objective onto a diffraction limited region of plasma membrane to create an observation volume on the order of 10^{-15} liters. As the fluorescent molecules are excited by the laser, the fluctuations in fluorescence intensity are recorded in real time, providing quantitative information about diffusion, number and brightness of the fluorescent molecules. Since the observation volume is not illuminated homogenously, the detected photon counts decrease as a fluorescent molecule moves away from the center of the observation volume. Thus, optimal positioning within the center of the observation volume is critical for accurate molecular brightness determination of plasma membrane proteins. In the present study, FCS measurements were made on the upper plasma membrane of HEK293 cells, or hippocampal neuronal cell body, transfected with fluorescent-tagged 5-HT_{2C} receptors. Positioning of the plasma membrane in the center of the observation volume was best achieved by scanning the sample along the Z-axis while simultaneously monitoring the photon counts per molecule. Three different fluorescent probes were used to ensure that the results obtained were not due to a unique photophysical property of the fluorescent probe, and to determine reproducibility of results. GFP and mCherry produced nearly identical diffusion coefficients for 5-HT_{2C} receptors, while the diffusion coefficient for YFP-tagged receptors was slightly faster. These results are similar to previously published diffusion coefficients for galanin, adenosine, and bradykinin receptors (30, 57, 58).

The diffusion rate of a membrane protein is related to the cubic root of the mass of the protein, such that an eight-fold change in a protein’s mass would be required to produce a two-fold change in the diffusion rate. It should be noted that the limit of resolution of the FCS technique is such that the diffusion rates of two proteins must differ by a factor of 1.6 or greater in order to be resolved by FCS (62). Therefore, diffusion rates can’t be used to differentiate between monomers, dimers or tetramers of a given protein.

Information about a protein’s oligomer status can be extracted by analyzing the amplitude of the fluctuations in fluorescence intensity obtained in an FCS experiment. PCH analysis uses the information encoded in the amplitude of the autocorrelation function to determine the average molecular brightness of the fluorescent molecules (40). PCH provides quantitative information about the number of fluorescent molecules and the number of photon counts per molecule. PCH analysis uses a 3D Gaussian approximation of the laser beam profile and Poisson statistics to predict what the molecular brightness of the fluorescent particle would be when it is at the center of the observation volume (40). The molecular brightness is proportional to the number of fluorescent molecules traveling together within a protein complex. Thus if a receptor monomer with a single fluorescent tag has a molecular brightness of X, then the molecular brightness of a receptor dimer carrying two fluorescent tags would be 2X, a tetramer would be 4X and so forth. FCS and PCH report the average molecular brightness of all fluorescent species present in the sample. Thus if a sample contained a mixture of two species of differing brightness (monomers and dimers in equilibrium for example), the brightness value would be an average based on the proportion of each species in the mixture (60).

Several different approaches were used to establish the molecular brightness of YFP and GFP monomers and dimers to serve as controls for determining the oligomeric state of 5-HT_{2C} receptors. To determine the molecular brightness of a monomer, HEK293 cells were transfected with plasmids containing monomeric GFP or YFP and FCS/PCH measurements were made within the cytosol of transfected cells. In a similar manner, the molecular brightness of a dimer was determined in HEK293 cells transfected with a plasmid containing two GFP linked together in tandem. BiFC between the N-terminal and C-terminal halves of YFP attached to the 5-HT_{2C} receptor was employed as an additional method to confirm 5-HT_{2C} receptor dimerization and as a control in the FCS/PCH studies. Co-expression of 5-HT_{2C}/N-YFP and 5-HT_{2C}/C-YFP resulted in successful recombination of YFP and reconstitution of plasma membrane fluorescence,
suggestive of 5-HT$_{2C}$ receptor dimerization. FCS and PCH measurements made on the plasma membrane of HEK293 cells co-expressing the 5-HT$_{2C}$/N-YFP + 5-HT$_{2C}$/C-YFP BiFC pair would be expected to yield a molecular brightness approximately half that observed for 5-HT$_{2C}$/YFP receptors. If 5-HT$_{2C}$ receptors are predominantly dimers then the molecular brightness of the BiFC pair should be similar to the monomeric YFP control. If 5-HT$_{2C}$ receptors form tetramers and higher order oligomers, then the molecular brightness of the BiFC pair would be predicted to be greater than monomeric YFP. As presented in table 2, the molecular brightness of the BiFC pair was half that of 5-HT$_{2C}$/YFP and similar to monomeric YFP. Thus, the result of the BiFC experiment is consistent with a dimeric structure of the 5-HT$_{2C}$ receptor.

To confirm and validate these results an additional control was added wherein HEK293 cells were co-transfected with 5-HT$_{2C}$/GFP and a three-fold excess of un-tagged 5-HT$_{2C}$ receptor. If 5-HT$_{2C}$ receptors are monomeric, then this co-transfection experimental design would have no effect on the observed molecular brightness of 5-HT$_{2C}$/GFP. However, if 5-HT$_{2C}$ receptors are dimeric, then this experimental design would result in the formation of 5-HT$_{2C}$ dimers in which only one protomer of the dimer has a GFP tag and the resulting molecular brightness would be half that observed for cells transfected with 5-HT$_{2C}$/GFP alone. If 5-HT$_{2C}$/GFP receptors form tetramers, then co-expression with a three-fold excess of un-tagged 5-HT$_{2C}$ receptor would be predicted to reduce the molecular brightness by greater than 50%. Additionally, if 5-HT$_{2C}$ receptors exist as an equal mixture of monomers and dimers, then the molecular brightness would be predicted to be reduced by one third, following co-expression with an excess of untagged receptor. The results presented in table 2 show that co-expression with excess untagged 5-HT$_{2C}$ receptor reduced the molecular brightness to half that observed for 5-HT$_{2C}$/GFP alone. Again, these results are consistent with a dimeric structure for the 5-HT$_{2C}$ receptor.

GPCR dimer/oligomer studies performed in recombinant cell systems have been criticized for high protein expression levels that are non-physiological and could possibly promote GPCR aggregation. Also, it has been reported that high concentrations of GFP or YFP can promote self assembly to form dimers/oligomers (63). Such conditions could lead to erroneous conclusions about the oligomeric size of GFP- and YFP-tagged receptors. One of the main advantages of the FCS/PCH technique is that it requires very low protein expression levels. The ability to monitor or track changes in fluorescence intensity as individual fluorescent molecules enter and leave the observation volume is dependent upon having very few molecules within the observation volume at any given time. As the concentration of fluorescent molecules increases, the ability to detect a significant change in fluorescence intensity as another molecule enters the observation volume is dramatically reduced. Therefore, low receptor expression levels are required in order to make valid FCS/PCH measurements. In addition, we employed an A206K mutation in the GFP tags, previously shown to eliminate GFP aggregation (50). Therefore, the possibility of non-specific receptor aggregation due to high expression levels and self assembly of fluorescent tags have been eliminated in the present study.

The results of our BiFC studies provide evidence for GPCR dimer formation during receptor biosynthesis in the ER/Golgi, consistent with our previous findings using FRET (11). Mutagenesis studies suggest that GPCR dimerization may be necessary for exit from the ER/Golgi and trafficking to the plasma membrane (12,13). If this is in fact the case, then GPCR monomers would not traffic to the plasma membrane, and the only way to have monomeric GPCR would be for dimers to dissociate into monomers once they reach the plasma membrane.

Recent studies using TIRF imaging of M1-muscarinic and formyl peptide receptors have suggested that dimers dissociate into monomers on the plasma membrane and exist in a monomer-dimer equilibrium (24, 25). However, the limit of resolution of TIRF imaging exceeds the dimensions of an individual GPCR. In the present study, if 5-HT$_{2C}$ receptors exist as a mixture of monomers and dimers, then the FCS/PCH results would have produced molecular brightness values that were in between that of the monomeric and dimeric controls, as PCH provides an estimate of the average molecular brightness of all fluorescent species present in the sample. Müller and
colleagues tested the sensitivity of the PCH method to resolve a mixture of two fluorescent species (60). They found an optimal ratio of 83% of the dimmer species and 17% of the brighter species for resolving the two species using a two component PCH model with reduced chi square equal to unity (60). In our experiments, two component PCH models testing for the presence of a mixture of monomers and dimers or dimers and tetramers, did not provide a better fit of the data than the one component PCH model. Reduced chi square values for the goodness of fit to a one component PCH model were close to unity, indicating the data were best described by a model predicting dimers and not a mixture of monomers and dimers, or dimers and tetramers. Sophisticated RET approaches and FRAP have been applied to detect the presence of higher order GPCR oligomers (22, 23, 64). While these studies have concluded that GPCR form tetramers, results of the TIRF imaging studies (24, 25) and our FCS/PCH results do not support this conclusion.

While there is a large body of evidence suggesting that class A GPCR may form and function as dimers/oligomers, there is no consensus as to their oligomeric size. Previous studies using co-IP, RET, TIRF and FRAP have produced conflicting results, even within the same sub-family of GPCR. For example, muscarinic receptors have been reported to be monomers (24), dimers (65, 66), and tetramers (22,63). Similarly, beta-adrenergic receptors have been reported to be monomers (67), dimers (18) and higher order oligomers (21, 64). In part, these conflicting results may be due to differences in the methods employed, variations in the sensitivity of the methods, and/or interpretation of the results. The application of live-cell methods with single molecule sensitivity, such as FCS and PCH, should be helpful in resolving this issue.

In conclusion, FCS with confocal microscopy provides a powerful method with single molecule sensitivity for determining the number and molecular brightness of fluorescent-tagged proteins as a measure of their oligomer size. In the present study, FCS and PCH brightness analyses yielded identical results. In HEK293 cells and hippocampal neurons the molecular brightness of YFP-tagged 5-HT$_{2C}$ receptors was twice that of the BiFC pair and monomeric YFP. The molecular brightness of GFP-tagged 5-HT$_{2C}$ receptors was twice that of monomeric GFP and similar to tandem GFP. Our data for plasma membrane 5-HT$_{2C}$ receptors were adequately described by a one component PCH model, for a single fluorescent species, with reduced chi square equal to unity. The FCS/PCH data are best described by a model in which plasma membrane 5-HT$_{2C}$ receptors exist as dimers. The dimeric structure was preserved following 5-HT binding and receptor activation. Therefore, it is concluded that the dimer represents the basic signaling unit. For 5-HT$_{2C}$, 5-HT$_4$ and 5-HT$_7$ receptors, binding to both protomers of the dimer is suggested to be required for maximal activation (8-10). Thus it is possible that bivalent ligands may have therapeutic potential.
REFERENCES

FCS analysis of GPCR oligomer size

35) Chen, Y., Wei, L., and Muller, J.D. (2003) PNAS 100, 15492-15497
Acknowledgments

This work was supported by National Institute of Health grant R21MH086796 to K.H.D.
FIGURE LEGENDS:

Figure 1: Confocal microscopy of HEK293 cells transfected with BiFC constructs. A) The N- and C-terminal halves of YFP were attached to the C-terminal end of 5-HT2C receptors and co-transfected (5-HT2C/N-YFP + 5-HT2C/C-YFP) into HEK293 cells. Plasma membrane YFP fluorescence, 20 hours post-transfection, is shown in the first panel. The second panel shows the DIC image and the third panel shows the fluorescence overlay on the DIC image. Red scale bar = 10µm. B) HEK 293 cells co-transfected with 5-HT2C/N-YFP + β2-adrenergic/C-YFP, imaged 20 hours post-transfection. C) HEK293 cells co-transfected with β2-adrenergic/N-YFP + β2-adrenergic/C-YFP, imaged 20 hours post-transfection. D) HEK293 cells co-transfected with 5-HT2C/N-YFP + 5-HT2C/C-YFP. Sixteen hours post-transfection YFP fluorescence is observed in the ER/Golgi.

Figure 2: Confocal microscopy of an isolated rat hippocampal neuron transfected with 5-HT2C/YFP and maintained in culture for one day. The insert shows the upper plasma membrane of the cell body and the location (+) where an FCS recording was made. Red scale bar = 10µm.

Figure 3: FCS recordings from the plasma membrane of hippocampal neurons expressing 5-HT2C/YFP and HEK293 cells expressing 5-HT2C/GFP or 5-HT2C/mCherry. A) Fluorescence intensity traces for one 10 second observation period. B) Biphasic FCS autocorrelation curves, generated from the intensity traces shown above. The blue line represents the autocorrelation of the observed fluorescence and the green line represents the fit to a two component model (for 5-HT2C/mCherry the colors are red and green, respectively). The fast component (measured in microseconds) is related to the photo-physical properties of the fluorescent probe, while the slower component (measured in milliseconds) represents the diffusion of fluorescent-tagged receptors in the plasma membrane. The diffusion rates are calculated from the midpoint of the decay of the autocorrelation curve. C) Residuals of the curve fit from the autocorrelation analysis. Dividing the average photon count rate (kHz) determined from the fluorescence intensity trace shown in A by the number of fluorescent molecules determined from the autocorrelation curve shown in B (calculated as in equation 3, methods, where G(0) = the Y intercept at the 1µs time point) predicts the average molecular brightness of the sample expressed as counts per second per molecule.

Figure 4: Photon counting histograms from FCS recordings made in the cytosol of HEK293 cells expressing a tandem, GFP-GFP dimeric construct (A) and the plasma membrane of HEK293 cells expressing 5-HT2C/GFP receptors (B). The inset shows the PCH on a linear scale. To generate a histogram, each 10 second fluorescence intensity trace (as illustrated in figure 3a) was broken down into one million 10µs intervals or bins (PCH bin time = 10µs). Histograms were constructed (using the PCH module in the Zeiss Aim 4.2 software) in which the number of 10µs bins was plotted on the y-axis and photon counts on the x-axis. The resulting histogram depicts the number of bins that registered 1,2,3…n photon counts during one 10 second observation period. The histograms show the average number of photon counts per 10µs bin time to be 1.2, equivalent to 120,000 counts per second. Dividing by the average number of molecules in the observation volume (7) yields an average molecular brightness of 17,143 counts per second per molecule. The residuals of the curve fit (shown in the lower panels) plot the number of bins on the y-axis and photon counts on the x-axis. The residuals show the deviation of the fit of the data to the selected model, providing a measure of how well the data fit the model. In this case the data were fit to a one component model for a single homogenous population of fluorescent tagged receptors (ie. dimers). The residuals of the curve fit are less than 2 standard deviations and are randomly distributed about zero, indicating that the data are a good fit for the selected model, with reduced chi square equal to unity.

Figure 5: Live-cell time course of beta-arrestin recruitment to the plasma membrane following activation of 5-HT2C receptors. HEK293 cells were co-transfected with beta-arrestin2/CFP (shown in green) and 5-HT2C/YFP (shown in red). Images were recorded prior to treatment with 5-HT (T=0), and 30 seconds (T=30) and 90 seconds (T=90) following application of 50nM 5-HT. Beta-arrestin is entirely cytosolic.
prior to 5-HT, and appears co-localized with 5-HT$_{2C}$ receptors on the plasma membrane 30 seconds and 90 seconds after 5-HT treatment (yellow plasma membrane in the merged image).
FCS analysis of GPCR oligomer size

Table 1: Diffusion coefficients for fluorescent-tagged 5-HT$_{2C}$ receptors expressed on the plasma membrane of hippocampal neurons and HEK293 cells. FCS data were best fit by a two component model with $\tau_{D1}$ representing the photo-physical properties of the fluorescent probe and $\tau_{D2}$ the diffusion of receptors within the plasma membrane. Data represent the mean +/- sem for the number of cells (N) as indicated.

<table>
<thead>
<tr>
<th>Hippocampal Neurons</th>
<th>$\tau_{D1}$ (µs)</th>
<th>$\tau_{D2}$ (ms)</th>
<th>Diffusion (µm$^2$/s)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT$_{2C}$/YFP</td>
<td>54.6 +/- 3.0</td>
<td>10.2 +/- 0.6</td>
<td>1.92 +/- 0.11</td>
<td>20</td>
</tr>
<tr>
<td>HEK293 Cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT$_{2C}$/YFP</td>
<td>50.5 +/- 2.9</td>
<td>16.9 +/- 0.4</td>
<td>1.16 +/- 0.03</td>
<td>20</td>
</tr>
<tr>
<td>5-HT$<em>{2C}$/N-YFP + 5-HT$</em>{2C}$/C-YFP BiFC</td>
<td>58.6 +/- 4.1</td>
<td>16.5 +/- 0.9</td>
<td>1.19 +/- 0.07</td>
<td>15</td>
</tr>
<tr>
<td>5-HT$_{2C}$/GFP</td>
<td>228 +/- 6.5</td>
<td>25.4 +/- 1.2</td>
<td>0.77 +/- 0.04</td>
<td>20</td>
</tr>
<tr>
<td>5-HT$_{2C}$/mCherry</td>
<td>59.8 +/- 4.1</td>
<td>25.9 +/- 0.9</td>
<td>0.76 +/- 0.03</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 2: Molecular brightness of fluorescent-tagged 5-HT$_{2C}$ receptors expressed on the plasma membrane of hippocampal neurons and HEK293 cells. Molecular brightness values were calculated from the FCS data by dividing the average photon count rate (obtained from the fluorescence intensity trace) by the number of fluorescent molecules (derived from the autocorrelation function) and are expressed as counts per second per molecule (CPSM). Molecular brightness determined by PCH was performed using the PCH module in the Zeiss Aim 4.2 software for a one component model and the resulting reduced chi square value is reported. Data represent the mean +/- sem for the number of cells indicated (N) from two or three independent transfection experiments.

<table>
<thead>
<tr>
<th>Hippocampal Neurons</th>
<th>Molecular Brightness (CPSM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurons</td>
<td>FCS</td>
</tr>
<tr>
<td>5-HT$_{2C}$/YFP</td>
<td>17,286 +/- 1,257</td>
</tr>
<tr>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>5-HT$_{2C}$/YFP</td>
<td>17,763 +/- 1,057</td>
</tr>
<tr>
<td>5-HT$<em>{2C}$/N-YFP + 5-HT$</em>{2C}$/C-YFP BiFC</td>
<td>9,466 +/- 1,791</td>
</tr>
<tr>
<td>monomeric YFP</td>
<td>9,403 +/- 349</td>
</tr>
<tr>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>5-HT$_{2C}$/GFP</td>
<td>17,751 +/- 426</td>
</tr>
<tr>
<td>5-HT$_{2C}$/GFP + 5-HT</td>
<td>18,149 +/- 1,074</td>
</tr>
<tr>
<td>Tandem GFP</td>
<td>19,294 +/- 703</td>
</tr>
<tr>
<td>5-HT$<em>{2C}$/GFP + 5-HT$</em>{2C}$(1:3)</td>
<td>9,003 +/- 314</td>
</tr>
<tr>
<td>monomeric GFP</td>
<td>9,266 +/- 211</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 3

A) Hippocampal Neuron 5-HT2C/YFP
   HEK293 5-HT2C/GFP
   HEK293 5-HT2C/mCherry

B) G(t) - Fit deviation
   τD1=59us
   τD2=11ms
   1e-0060 0.000010 0.0001 0.001 0.01 1
   τD1=195us
   τD2=24ms
   1e-0060 0.000010 0.0001 0.001 0.01 1
   τD1=54us
   τD2=26ms
   1e-0060 0.000010 0.0001 0.001 0.01 1

Fit range: 1.00 μs to 1.05 s
Figure 4

A) Tandem GFP

B) 5-HT2C/GFP
Figure 5

beta-arrestin/CFP + 5-HT2C/YFP Merge

T=0 - 5-HT

T=30s +5-HT

T=90s +5-HT
Oligomer size of the serotonin 5-HT2C receptor revealed by fluorescence correlation spectroscopy with photon counting histogram analysis: evidence for homodimers without monomers or tetramers.

Katharine Herrick-Davis, Ellinor Grinde, Tara Lindsley, Ann Cowan and Joseph E. Mazurkiewicz

*J. Biol. Chem.* published online May 16, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M112.350249

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/early/2012/05/16/jbc.M112.350249.full.html#ref-list-1