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Nanoscale organization of β_2 -adrenergic receptor-Venus fusion protein domains on the surface of mammalian cells

Dusan Vobornik^a, Yanouchka Rouleau^a, Jennifer Haley^a, Mahmud Bani-Yaghoub^b, Rod Taylor^a, Linda J. Johnston^{a,*}, John Paul Pezacki^{a,*}

^a Steacie Institute for Molecular Sciences, National Research Council Canada, Ottawa, ON, Canada K1A 0R6

^b Institute for Biological Sciences, National Research Council Canada, Ottawa, ON, Canada K1A 0R6

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ABSTRACT

Adrenergic receptors are a key component of nanoscale multiprotein complexes that are responsible for controlling the beat rate in a mammalian heart. We demonstrate the ability of near-field scanning optical microscopy (NSOM) to visualize β_2 -adrenergic receptors (β_2 AR) fused to the GFP analogue Venus at the nanoscale on HEK293 cells. The expression of the β_2 AR-Venus fusion protein was tightly controlled using a tetracycline-induced promoter. Both the size and density of the observed nanoscale domains are dependent on the level of induction and thus the level of protein expression. At concentrations between 100 and 700 ng/ml of inducer doxycycline, the size of domains containing the β_2 AR-Venus fusion protein appears to remain roughly constant, but the number of domains per cell increase. At 700 ng/ml doxycycline the functional receptors are organized into domains with an average diameter of 150 nm with a density similar to that observed for the native protein on primary murine cells. By contrast, larger micron-sized domains of β_2 AR are observed in the membrane of the HEK293 cells that stably overexpress β_2 AR-GFP and β_2 AR-eYFP. We conclude that precise chemical control of gene expression is highly advantageous for the use β_2 AR-Venus fusion proteins as models for β_2 AR function. These observations are critical for designing future cell models and assays based on β_2 AR, since the receptor biology is consistent with a relatively low density of nanoscale receptor domains.

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Introduction

The beating rate in the mammalian heart is strongly influenced by the binding of catecholamines to β -adrenergic G-protein coupled receptors (β ARs) in cardiac myocytes, initiating an adrenergic response [1–3]. The signaling cascade initiated by catecholamine binding requires the association of β ARs into multiprotein complexes called signalosomes [4] that include the α , β and γ G-protein subunits. Changes in the molecular composition of signalosomes are associated with receptor desensitization, sequestration of the receptor to subcellular membranous compartments and internalization, all of which strongly influence β AR function [5–11]. Signaling has also been shown to depend on differential interactions with scaffolding proteins in signaling complexes [12]. Recently we have used near field scanning optical microscopy (NSOM) to demonstrate that functional receptors are organized into multi-protein domains of ~140 nm average diameter on murine neonatal and embryonic cardiac myocytes [13]. Colocalization experiments in

these primary cells at the nanometer scale show that 15–20% of receptors are pre-associated in caveolae, an important component of signalosomes [13]. β AR signaling represents an important pharmacological target [14] and many assays have been developed to aid in the drug discovery process [15–18]. Herein we investigate the conditions required for mimicking the nanoscale distributions and local environment observed in primary cells from the mammalian heart using a conditionally expressed fusion protein of β_2 AR in HEK293 cells.

Fusion proteins of β ARs have provided useful tools for studies of adrenergic receptor biology and the development of assays for ligand discovery based on bioluminescence and Forster resonance energy transfer (BRET and FRET, respectively) [2,15–24]. Functional fusion proteins of β_2 ARs have been accomplished with FLAG tags for immunofluorescence and immunoprecipitation experiments as well as green fluorescent proteins (GFPs) and their analogues [15–18,25]. Inducible expression of GPCRs has also been achieved allowing for precise chemical control of expression levels in mammalian cells [26,27]. We have adapted one of the human β_2 AR-GFP fusion proteins to contain the “Venus” yellow fluorescent protein which was optimized for biophysical studies [28]. We placed the human β_2 AR-Venus fusion protein under tetracy-

* Corresponding authors. Fax: +1 613 941 8447 (J.P. Pezacki).

E-mail addresses: Linda.Johnston@nrc-cnrc.gc.ca (L.J. Johnston), John.Pezacki@nrc-cnrc.gc.ca (J.P. Pezacki).

cline-dependent expression using the “Tet-On” system in order to have precise chemical control of membrane protein expression so that we could correlate expression levels with nanoscale clustering on the cell surface.

Materials and methods

Cell culture. HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (NorthBio, Toronto, ON), penicillin (100 IU/ml), and streptomycin (100 mg/ml) (Invitrogen) under standard culture conditions (37 °C, 5% CO₂). The HEK 293^{Tet-On} cells, which stably express the reverse transactivator (rTA) from the Tet-On inducible system, were grown in complete DMEM with 800 µg/ml G418 (Invitrogen). The β_2 AR FRET cells stably expressing β_2 AR-GFP² and β_2 AR-eYFP were grown in DMEM supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 mg/ml), 300 µg/ml G418 and 300 µg/ml Zeocin (Invitrogen) under standard culture conditions (37 °C, 5% CO₂).

Eukaryotic expression vector. The pTRE β_2 AR-Venus construct was created by cutting out the β_2 -adrenergic receptor coding sequence

including the stop signal from pRL-CMV- β_2 AR-RLuc, which was a generous gift of Dr. Bouvier (Université de Montréal), using the NheI restriction enzyme. It was then subcloned in pTREhyg, supplied in the BDTM Tet-on gene expression system kit (Clontech Laboratories Inc, Mountain View, CA), in the NheI site. The Venus coding sequence was amplified by Polymerase Chain Reaction (PCR), using pVS1 plasmid, which was a generous gift of Dr. Xie (Harvard University) as a template. The restriction sites NotI and Sall were added, respectively, to the C- and N-terminus of Venus. The reverse primer sequence was TATTGTCGACTTACTTGTAC AGCTCGTCC and the forward primer sequence was ATAATAAAG CCGCCGCTAATGGTGAGCAAGGGCG. The Venus gene was then subcloned in frame with β_2 AR in the pTREhyg backbone. The p β_2 AR-GFP² construct was created by cutting out the β_2 AR coding sequence as described earlier and it was then subcloned in pGFP2-N3 (PerkinElmer Life and Analytical Sciences, Boston, MA). The p β_2 AR-eYFP construct was a generous gift of Dr. Bouvier (Université de Montréal).

Inducible system. The BDTM Tet-on gene expression system kit was used to express wild type β_2 AR fused to Venus, an YFP variant in HEK 293 cells under the control of a doxycycline inducible pro-

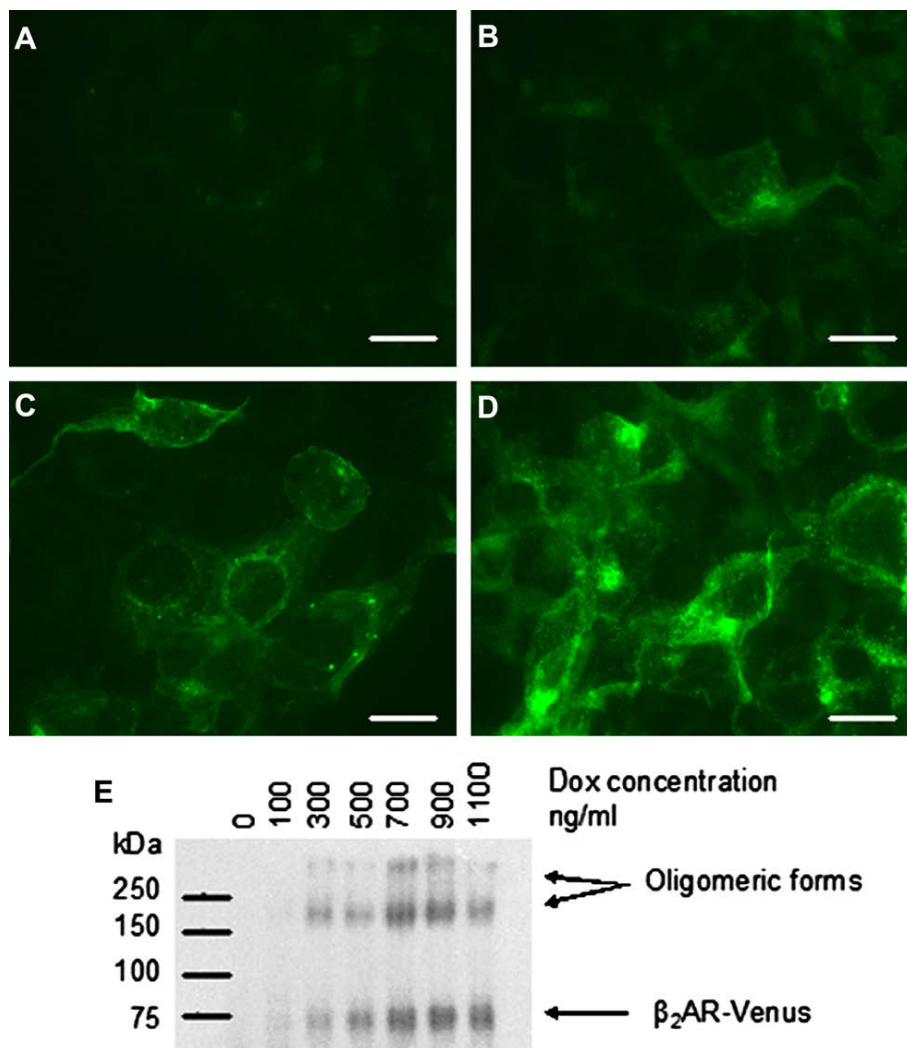


Fig. 1. Fluorescence microscopy images of (A) L9 (HEK293^{Tet-On}) cells not treated with Dox; (B) L9 cells transiently transfected with β_2 AR-Venus gene but not treated with Dox; (C) L9 cells transfected with β_2 AR-Venus gene and treated with 100 ng/ml Dox; (D) L9 cells transfected with β_2 AR-Venus gene and treated with 700 ng/ml Dox with scale bars representing 10 µm; (E) Western blot of the immunoprecipitated β_2 AR-Venus at various Dox concentrations.

moter. The HEK 293^{Tet-on} cell line was developed from wild-type HEK 293 cells by transfection with the regulator pTet-On plasmid (5 μ g) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), as instructed by the manufacturer. Colonies were isolated and transferred into 10 cm tissue culture dishes in the presence of 800 μ g/ml of G418 in complete DMEM. G418-resistant clones were expanded and screened by luciferase assay using the pTRE2hyg-Luc reporter plasmid, which expresses a luciferase gene under the control of the tetracycline-response element (TRE).

Imaging of fixed cells. To examine the effect of different doxycycline (Dox) concentrations on the expression level of β_2 AR-Venus, Lab-Tek II 8-well chambered slides (Nalgene Nunc, Rochester, NY) were seeded with HEK 293^{Tet-on} cells. The cells were then transfected with 0.1 μ g pTRE β_2 AR-Venus plasmid per

well. Four hours post-transfection growth media with 20% FBS was added and the cells were allowed to recover for 24 h. Following the 24 h recovery period the media was replaced with growth media with or without varying concentrations of Dox to induce expression of β_2 AR-Venus. Twenty-four hours post-induction the cells were washed once with 1 \times PBS pH 7.4, fixed with 3.7% formaldehyde for 30 min at 4 $^{\circ}$ C and washed 3 times with 1 \times PBS to remove the excess formaldehyde. Slides were cover slipped using anti-fade mounting medium (pH 8.0; 1 mg *p*-phenylenediamine, PBS, 90% glycerol). Imaging was done on an Olympus IX81 microscope using the Olympus Plan Apochromat 60 \times , 1.45 NA oil immersion objective and the Blue Argon (488 nm) laser. Images were captured using a Cool Snap ES camera (Roper Scientific Photometrics, Tucson, AZ).

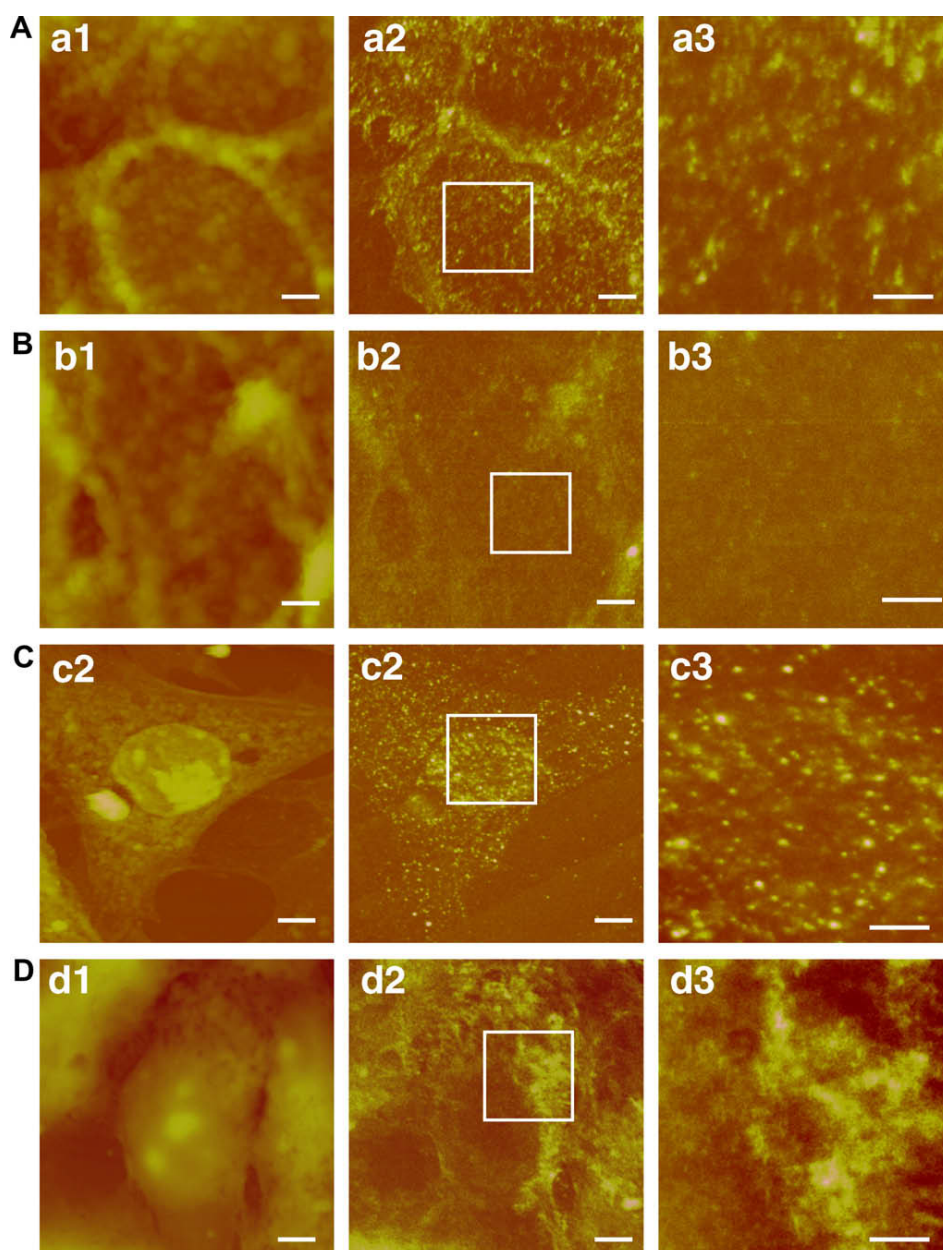


Fig. 2. Representative NSOM images of (A) HEK293^{Tet-on} cells transfected with β_2 AR-Venus and treated with 700 ng/ml of Dox, (B) control HEK293^{Tet-on} cells, not treated with Dox, (C) cardiac myocytes with immuno-labeled β_2 AR, and (D) HEK293 cells stably expressing β_2 AR-eYFP and β_2 AR-GFP²; a1, b1, c1 and d1 show topography with scale bars at 5 μ m; a2, b2, c2 and d2 are matching fluorescence images of the same area; a3, b3, c3 and d3 are zoom-in images corresponding to the white squares in a2, b2, c2 and d2 with scale bars of 2 μ m and intensity scales of 30 kcounts/s.

NSOM. Bent NSOM probes were prepared from high GeO₂-doped fibers with a core diameter of 3 μm via a two-step chemical etching method followed by aluminum deposition and focused ion-beam milling to produce a flat circular aperture [29]. The probes used in the present work had aperture diameters of roughly 100 nm (estimated from SEM images and by imaging 40-nm dye-labeled polymer microspheres). The estimated spring constant for these probes is ~100 Nm⁻¹ [29]. NSOM experiments were carried out on a combined AFM/NSOM microscope based on a Digital Instruments Bioscope mounted on an inverted fluorescence microscope (Zeiss Axiovert 100). Cellular imaging was carried out using 488 nm excitation. The NSOM cellular imaging procedure is described in detail previously [13,30].

For experiments in which the effect of Dox concentration was examined in HEK293 cells, at least three different 40 × 40 μm² areas and three 10 × 10 μm² areas were imaged for each sample at a constant laser power. HEK293 cells expressing β₂AR-GFP² and β₂AR-eYFP were imaged with a probe with a similar aperture diameter, but required significantly lower laser excitation intensity to achieve similar NSOM fluorescence signals. We verified that the NSOM probe aperture remained unchanged throughout the experiments by scanning 40 nm fluorescent spheres before and after the experiments. Cluster size analysis was performed using original non-processed NSOM images with custom-made software that

determines the number of clusters and their location in the image, as well as their full width at half maximum and maximum intensity, as previously described [13,30]. Images shown in the [Supporting information](#) demonstrate the ability of the software to accurately count most of the clusters; however, there are some diffuse patches of fluorescence which cannot be distinguished from the background. Representative histograms of cluster size for L9 cells transfected with the β₂AR-Venus gene and treated with 100 and 700 ng Dox are shown in Fig. 3. Note that the smallest feature size is limited by the size of the probe aperture used; cluster sizes reported represent a convolution of probe aperture and feature size. The total intensities for individual clusters were calculated from the cluster diameter and maximum intensity, assuming a Gaussian-shaped intensity profile.

Results and discussion

We created a stable cell line containing the reverse tetracycline-controlled transactivator (rtTA) gene, carefully selecting a clone that had the lowest background expression and best sensitivity from a response plasmid which expressed luciferase under control of the tetracycline-response element (TRE) when treated with doxycycline (Dox) (see Fig. S1, [supporting information](#)). This allowed us to have precise chemical control of the expression levels

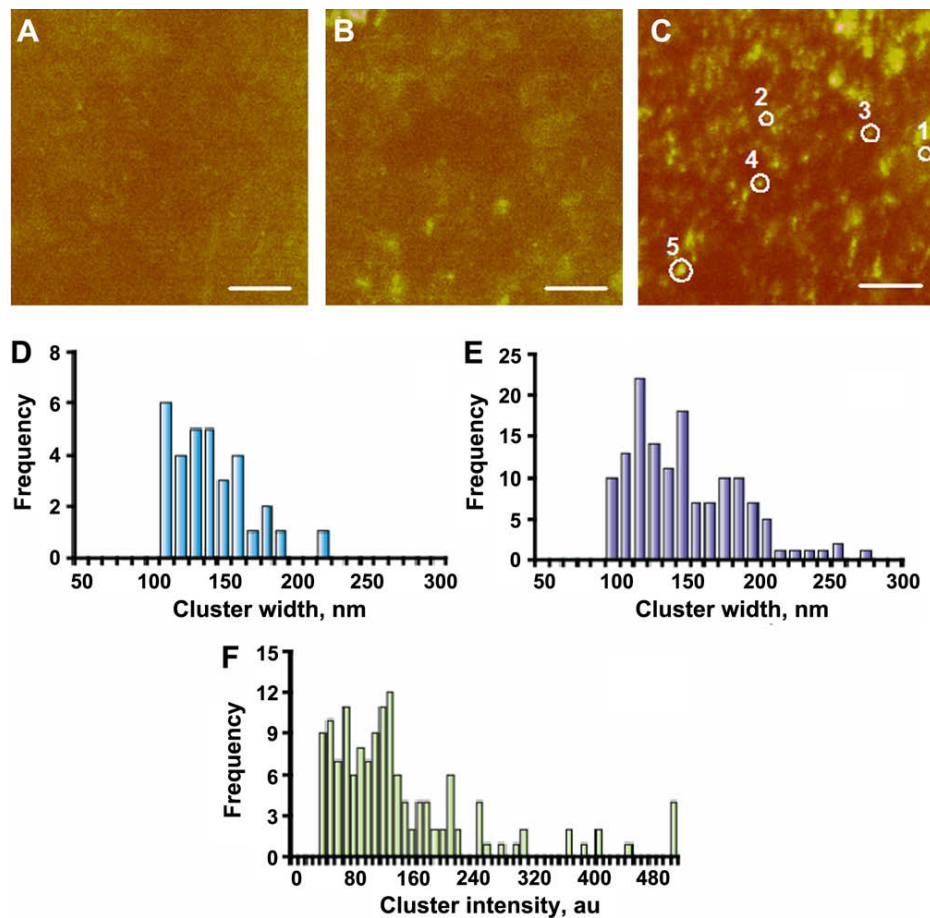


Fig. 3. Representative NSOM fluorescence images of (A) control L9 cells (HEK293^{Tet-On}) transfected with the response plasmid containing β₂AR-Venus but not treated with Dox, (B) L9 cells expressing β₂AR-Venus upon treatment with 100 ng/ml Dox, (C) L9 cells expressing β₂AR-Venus treated with 700 ng/ml Dox. Scale bars correspond to 2 μm, and the vertical range is 20 kcounts/s. The histograms in (D,E) show cluster size distributions for images (B,C), with average cluster widths of 130 and 150 nm, respectively. Note that the measured cluster widths represent a convolution of probe aperture and feature size. Histogram (F) shows the distribution of total integrated fluorescence intensity for the individual clusters in image (C), with the last bin representing clusters of intensity >500. If we assume that the least intense clusters in the first bin represent β₂AR-Venus receptor dimers then estimates of 10 and 30 proteins for clusters of average and maximum intensity are obtained.

of the β_2 AR-venus fusion protein which was also placed in a response plasmid under TRE control in HEK293 cells stably transfected with the rtTA gene (L9 clone). Using fluorescence microscopy and Western blotting techniques, we established the tight control over β_2 AR-Venus fusion protein expression (Fig. 1). The fluorescence images and western blot results confirm that the β_2 AR-Venus fusion protein is not expressed in untreated cells; the expression level is low at 100 ng/ml Dox but increases steadily as the Dox concentration is increased to 700 ng/ml. Using ELISA assays on intact cells we show that at 700 ng/ml of Dox the β_2 AR-Venus protein is present at approximately the same physiologically relevant levels on the L9 clone as those on H9C2 rat cardiac myocyte cells (Fig. S2, supporting information). We observed that there was significant endogenous β_2 AR expression in the L9 clone, ~40% relative to the H9C2 cells; however, these are not fluorescent.

We used NSOM to investigate the expression of β_2 AR on L9 (HEK293^{Tet-On}) cells that were transfected with the response plasmid containing β_2 AR-Venus fusion protein at various concentrations of the inducer Dox. Using ~100 nm diameter aperture bent fiber NSOM probes operating in the tapping mode[™] and fluorescence microscopy, we examined the intensity and distribution of β_2 AR-Venus (Fig. 2). We observed distinct fluorescent patches on the surfaces of cells at Dox concentrations between 100 and 800 ng/ml, with fluorescence signals increasing in a similar trend to the level of protein expression shown in Fig. 1. Representative NSOM topography and fluorescence images are shown in Fig. 2A for cells treated with 700 ng/ml Dox. The β_2 AR-Venus is localized in many small clusters on the cell surface. Control experiments in which cells that were not transfected or were transiently transfected but not treated with Dox gave much lower signal intensities, as illustrated by the images shown in Fig. 2B for untransfected and untreated cells. Note that the larger image in Fig. 2b2 shows several diffuse patches of fluorescence that correlate with higher regions in the topographic scan (Fig. 2b1). However, the low signal intensity in the zoomed fluorescence image (Fig. 2b3) indicates minimal signal due to either background fluorescence or topography-related artifacts [13]. An additional control experiment for untransfected cells that were treated with 700 ng/ml Dox did not show a significant level of fluorescence signal (Fig. S3, supporting information).

The receptor distribution for cells treated with 700 ng/ml Dox is qualitatively similar to our previous observations of β AR localization within nanodomains in primary cells, as illustrated by the NSOM images for immunostained β_2 AR in cardiac myocytes (Fig. 2C). However, both of these results differ significantly from the NSOM images for cells stably overexpressing β_2 AR-GFP² and β_2 AR-eYFP in HEK293 cells (Fig. 2D), which show large diffuse patches of fluorescence, rather than small nanoscale clusters. As noted above, ELISA assays conducted on intact cells indicate that the levels of receptor at the cell membrane for cells treated with 700 ng/ml Dox are quantitatively similar to those on H9C2 cardiac myocytes where β ARs are localized within nanodomains. By contrast, HEK293 cells stably overexpressing β_2 AR-GFP² and β_2 AR-eYFP display at least 4-fold higher expression (Fig. S2, supporting information). Similarly, we have observed very large patches for other overexpressed GFP-fused receptors by NSOM rather than small clusters, further highlighting the importance of the development of inducible systems for the study of membrane protein biochemistry at the nanoscale.

Next we analyzed the cluster size and density of β_2 AR-Venus fusion proteins at low and high concentrations of Dox utilizing methods described previously [13,30]. The cluster densities increased with increasing Dox concentration as shown by the images in Fig. 3A–C for transfected cells treated with 0, 100, and 700 ng/ml Dox. Supplementary Fig. S4 shows images in which all counted clusters are marked for the Dox treated cells; note that the data

in Fig. 3C are displayed so that small weak clusters are visible, whereas at larger intensity scales it becomes evident that some of the larger features in the upper right corner of the image represent several smaller clusters. At 700 ng/ml Dox the sizes for the small roughly circular clusters vary in diameter from 100 nm to ~280 nm with an average of 150 nm (Fig. 3C and E) and the cluster density is 1.4 features/ μm^2 . The cluster size for 100 ng/ml Dox (Fig. 3B and D) is similar (average of 130 nm) but the cluster density is considerably lower at 0.4 features/ μm^2 . Note that there are also some areas of weak, diffuse signal at the lower Dox concentration that resemble the background observed in the untreated cells (Fig. 3A). The similar cluster size observed for high and low cluster densities indicates that there may be a biological preference for localization of β_2 AR receptors in ~150 nm diameter domains or clusters. The measured cluster density of 1.4 features/ μm^2 for cells treated with 700 ng/ml Dox is similar to the value of ~1.0 features/ μm^2 observed for β_2 AR on H9C2 cells as well as primary murine cells [13,30].

The clusters of β_2 AR-Venus have a range of intensities as shown by the histogram in Fig. 3F. By making the assumption that the clusters with the lowest total integrated intensity correspond to β_2 AR dimers, we estimate that the clusters in Fig. 3C contain from 2 to ~60 individual β_2 AR-Venus proteins; clusters with an average total intensity correspond to ~10 proteins. Because we observe significant levels of background β_2 AR expression in the HEK293^{Tet-On} cells, we cannot rule out that these features are made up of mixed receptor types (i.e. both β_2 AR and β_2 AR-Venus). Evidence from previous studies suggests that β_2 ARs translocate to the cellular membrane as dimers [31,32], although recently it has been demonstrated that monomeric GPCRs can activate G proteins [20]. If, in fact the lowest intensity features represent β_2 AR monomers then the estimates of individual proteins in Fig. 3 will range from 1 to ~30 β_2 AR-Venus proteins. These estimates are also similar to our previous estimates of 12–72 receptors/cluster for clusters ranging in diameter from 120 to 160 nm in immunostained primary cells [13,30].

Conclusions

In summary, we have demonstrated that NSOM fluorescence imaging of β_2 AR-Venus fusion membrane proteins is possible. Further we show that precise chemical control of receptor expression is highly advantageous to study the spatial and temporal localization of the receptor on the surfaces of mammalian cells. We observed that the cluster size and density and the numbers of receptor/cluster for β_2 AR-Venus in the “Tet-On” inducible system are very similar to our previous observations for immunostained β_2 AR in primary neonatal cells. This suggests that the inducible expression system gives a receptor distribution that is representative of the physiological state of the receptors on primary cells. The number of membrane protein clusters can be controlled using different Dox concentrations. However, it appears that the average size of clusters remains roughly constant with increasing Dox concentrations even though the number and density of clusters increase. The latter suggests that there is a strongly preferred membrane local environment for the β_2 AR at physiological protein expression levels. This is an important consideration for the design of new assays for β_2 AR function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.bbrc.2009.02.144.

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