Nanoscale organization of $\beta_2$-adrenergic receptor-Venus fusion protein domains on the surface of mammalian cells

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A B S T R A C T
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 $\beta_2$-adrenergic receptors ($\beta_2$AR) fused to the GFP analogue Venus at the nanoscale on HEK293 cells. The expression of the $\beta_2$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 $\beta_2$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 $\beta_2$AR are observed in the membrane of the HEK293 cells that stably overexpress $\beta_2$AR-GFP and $\beta_2$AR-eYFP. We conclude that precise chemical control of gene expression is highly advantageous for the use $\beta_2$AR-Venus fusion proteins as models for $\beta_2$AR function. These observations are critical for designing future cell models and assays based on $\beta_2$AR, since the receptor biology is consistent with a relatively low density of nanoscale receptor domains.

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I n t r o d u c t i o n

The beating rate in the mammalian heart is strongly influenced by the binding of catecholamines to $\beta$-adrenergic G-protein coupled receptors ($\beta$ARs) in cardiac myocytes, initiating an adrenergic response [1–3]. The signaling cascade initiated by catecholamine binding requires the association of $\beta$ARs into multiprotein complexes called signalosomes [4] that include the $\alpha$, $\beta$ and $\gamma$ 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 $\beta$AR function [5–11]. Signaling has also been shown to depend on differential interactions with scaffolding proteins in signal 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]. $\beta$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 $\beta_2$AR in HEK293 cells.

Fusion proteins of $\beta$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 $\beta$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 $\beta_2$AR-GFP fusion proteins to contain the "Venus" yellow fluorescent protein which was optimized for biophysical studies [28]. We placed the human $\beta_2$AR-Venus fusion protein under tetrycy-

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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 293Tet-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 b₂AR FRET cells stably expressing b₂AR-GFP and b₂AR-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 pTREb₂AR-Venus construct was created by cutting out the b₂-adrenergic receptor coding sequence including the stop signal from pRL-CMV-b₂AR-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 BD™ 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 TATTGTCGACTTACTTGTCAGCTCGTCC and the forward primer sequence was ATAATAAAGCGGCCGCTAATGGTGAGCAAGGGCCG. The Venus gene was then subcloned in frame with b₂AR in the pTREhyg backbone. The pB2AR-GFP construct was created by cutting out the b₂AR coding sequence as described earlier and it was then subcloned in pGFP2-N3 (PerkinElmer Life and Analytical Sciences, Boston, MA). The pB2AR-eYFP construct was a generous gift of Dr. Bouvier (Université de Montréal).

**Inducible system.** The BD™ Tet-on gene expression system kit was used to express wild type b₂AR fused to Venus, an YFP variant in HEK 293 cells under the control of a doxycycline inducible pro-
The HEK 293Tet-on cell line was developed from wild-type HEK 293 cells by transfection with the regulator pTet-On plasmid (5 μg) 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 μg/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 β2AR-Venus, Lab-Tek II 8-well chambered slides (Nalgene Nunc, Rochester, NY) were seeded with HEK 293Tet-on cells. The cells were then transfected with 0.1 μg pTREβ2AR-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 β2AR-Venus. Twenty-four hours post-induction the cells were washed once with 1× PBS pH 7.4, fixed with 3.7% formaldehyde for 30 min at 4°C and washed 3 times with 1× 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×, 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).

![Representative NSOM images](image_url)
NSOM. Bent NSOM probes were prepared from high GeO$_2$-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 N m$^{-1}$ [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 µm$^2$ areas and three 10 × 10 µm$^2$ areas were imaged for each sample at a constant laser power. HEK293 cells expressing β$_2$AR-GFP$^2$ and β$_2$AR-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 β$_2$AR-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) transfected with the response plasmid containing β$_2$AR-Venus but not treated with Dox, (B) L9 cells expressing β$_2$AR-Venus upon treatment with 100 ng/ml Dox, (C) L9 cells expressing β$_2$AR-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 β$_2$AR-Venus receptor dimers then estimates of 10 and 30 proteins for clusters of average and maximum intensity are obtained.](https://example.com/image-url)
of the β2AR-Venus fusion protein which was also placed in a response plasmid under TRE control in HEK293 cells stably transfec-
ted with the rtTA gene (L9 clone). Using fluorescence microscopy and Western blotting techniques, we established the tight control over β2AR-Venus fusion protein expression (Fig. 1). The fluores-
cence images and western blot results confirm that the β2AR-Ve-
nus 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 as-
says on intact cells we show that at 700 ng/ml of Dox the β2AR-Ve-
nus protein is present at approximately the same physiologically relevant levels on the L9 clone as those on H9c2 rat cardiac myo-
cyte cells (Fig. S2, supporting information). We observed that there was significant endogenous β2AR expression in the L9 clone, ∼40% relative to the H9c2 cells; however, these are not fluorescent.

We used NSOM to investigate the expression of β2AR on L9 (HEK293Tet-On) cells that were transfected with the response plas-
mid containing β2AR-Venus fusion protein at various concentra-
tions 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 β2AR-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 β2AR-Venus is localized in many small clusters on the cell surface. Control experiments in which cells that were not transfected or were transiently trans-
fected 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. 2B shows sev-
eral diffuse patches of fluorescence that correlate with higher re-
gions 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 topogra-
phy-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 βAR localiza-
tion within nanodomains in primary cells, as illustrated by the NSOM images for immunostained β2AR in cardiac myocytes (Fig. 2C). However, both of these results differ significantly from the NSOM images for cells stably overexpressing β2AR-GFPα and β2AR-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 βARs are localized within nanodomains. By con-
trast, HEK293 cells stably overexpressing β2AR-GFPα and β2AR-eYFP display at least 4-fold higher expression (Fig. S2, supporting information). Similarly, we have observed very large patches for other overexpressed GPCR-fused receptors by NSOM rather than small clusters, further highlighting the importance of the develop-
ment of individual systems for the study of membrane protein bio-
chemistry at the nanoscale.

Next we analyzed the cluster size and density of β2AR-Venus fusion proteins at low and high concentrations of Dox utilizing meth-
ods 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 repre-
sent 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/μm². The cluster size for 100 ng/ml Dox (Fig. 3B and D) is similar (average of 130 nm) but the cluster den-
sity is considerably lower at 0.4 features/μm². Note that there are also some areas of weak, diffuse signal at the lower Dox concentra-
tion 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 β2AR receptors in ∼150 nm diameter domains or clusters. The measured cluster density of 1.4 features/μm² for cells treated with 700 ng/ml Dox is similar to the value of ∼1.0 features/
μm² observed for β2AR on H9c2 cells as well as primary murine cells [13,30].

The clusters of β2AR-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 β2AR dimers, we estimate that the clusters in Fig. 3C contain from 2 to ∼60 individual β2AR-Venus proteins; clusters with an average total intensity correspond to ∼10 proteins. Because we observe sig-
nificant levels of background β2AR expression in the HEK293Tet-On cells, we cannot rule out that these features are made up of mixed receptor types (i.e. both β2AR and β2AR-Venus). Evidence from previous studies suggests that β2ARs translocate to the cellular mem-
brane as dimers [31,32], although recently it has been demonstrated that monomeric GPCRs can activate G proteins [20]. In fact, the lowest intensity features represent β2AR monomers whereas at higher intensity scales it becomes evident that some of the larger features in the upper right corner of the image repre-
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μm² observed for β2AR on H9c2 cells as well as primary murine cells [13,30].

Conclusions

In summary, we have demonstrated that NSOM fluorescence imaging of β2AR-Venus fusion membrane proteins is possible. Further we show that precise chemical control of receptor expression is highly advantageous to study the spatial and tem-
poral 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 β2AR-Venus in the “Tet-On” inducible system are very similar to our previous observations for immunostained β2AR in primary neonatal cells. This suggests that the inducible expression system gives a receptor distribu-
tion that is representative of the physiological state of the recep-
tors 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 con-
stant with increasing Dox concentrations even though the num-
ber and density of clusters increase. The latter suggests that there is a strongly preferred membrane local environment for the β2AR at physiological protein expression levels. This is an important consideration for the design of new assays for β2AR 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.

References


