**Supplementary Figure S1.** Isoproterenol stimulation of HA-β1- and HA-β2AR transfected H9c2 cells. (A) Unstimulated H9c2 cells expressing high levels of HA-β1- (top) and HA-β2AR (bottom) are shown in transmission (left) and after immunostaining (right). In unstimulated cells, both HA-β1- and HA-β2AR are located at the cell surface. (B) Stimulation with 10 μM isoproterenol for 10 minutes results in retention of HA-β1AR at the cell surface (top) and internalization of HA-β2AR (bottom). Cells were immunostained with anti-HA antibody and Alexa Fluor 488 labelled secondary antibody. (C) Unstimulated H9c2 cells expressing low levels (as used for SPT) of HA-β1- (top) and HA-β2AR (bottom) was also retained at the cell surface. Cells were labelled with Qdots (as used for SPT) and location of receptor was assessed by acid-wash (pH 3, Haggie et al., 2006) that rapidly removes accessible (cell surface) antibody complexes used to label HA-tagged receptors. Images are shown PRE (left) and POST (right) acid wash. (D) Stimulation with 10 μM isoproterenol for 10 minutes results in retention of HA-β1AR at the cell surface (top, receptors are removed by acid wash) and internalization of HA-β2AR (bottom, a significant proportion of receptors are not removed by acid wash). Scale bar in A and B, 10 μm. Scale bar in C and D, 5 μm.

**Supplementary Figure S2.** Specificity of Qdot labelling. H9c2 cells were transfected with HA-β1AR (left) and HA-β2AR (right) and labelled with anti-HA antibody, biotin conjugated Fab fragment and streptavidin conjugated Qdots emitting at 655 nm (as used for SPT). Qdots were imaged by fluorescence (top panels) and cells were imaged with transmitted light (bottom panels). Cell outlines in fluorescence images were traced from the transmission images to aid identification of labelled and unlabelled cells. The cells outlines in white were labelled with Qdots whereas proximal cells outlined in red and blue were not Qdot-labelled (and presumably untransfected). Images were acquired using the full CCD area (80 μm × 80 μm at 100× magnification).

**Supplementary Figure S3.** Fluorescence imaging of HA-β2AR labelled with anti-HA Fab fragments and DyLight 649-conjugated secondary Fab fragment. Individual frames from image sequences are shown. Data was acquired continuously at 10 fps with 100 ms exposures for 20 seconds. Labelled receptors are highlighted with colored circles and for the highlighted receptors, no photobleaching was observed prior to 15.5 seconds. Scale bar, 2 μm.

**Supplementary Figure S4.** Single particle tracking of HA-β1AR and HA-β2AR with mutated PDZ-binding domains. H9c2 cells were transfected with (A) HA-β1AR-V477A and (B) HA-β2AR-L413A expressing plasmids (using conditions as described for the wild-type HA-βARs) and receptors were labelled with 655 nm Qdots (as described for the wild-type HA-βARs). Representative trajectories (left) and cumulative distributions (right) of D (first panel) and range (second panel) are shown. Control data is shown in black. Cumulative distributions for mutated receptor constructs represent data from 121–131 trajectories derived from 15–20 cells.
Supplementary Figure S5. Dominant-negative affects of CAV3-P104L-GFP / mCherry expression on the localization of CAV3 interacting proteins. (A) Schematic representation of the image analysis method used to calculate CAV3 levels in non-Golgi regions of cells expressing (CAV3-P104L-GFP+) and not expressing (CAV3-P104L-GFP-) CAV3-P104L-GFP. Background subtracted, area integrated fluorescence intensities were calculated in cellular regions depicted by boxes. (B) Additional fluorescence micrographs of H9c2 cells expressing CAV3-P104L-GFP (top) immunostained for CAV3 (identifying endogenous CAV3 and CAV3-P104L-GFP, bottom). In both image sets, cells that express and do not express CAV3-P104L-GFP are shown (see also Figure 4). (C) Expression of CAV3-P104L-GFP did not alter HA-β2AR expression pattern. Two sets of example images are shown for transfected cells depicting CAV3-P104L-GFP localization (top) and HA-β2AR immunostaining (bottom). Adjacent cells expressing HA-β2AR but not CAV3-P104L-GFP and not expressing CAV3-P104L-GFP or HA-β2AR (denoted by *) are also shown. (D) CAV3-P104L-mCherry expression alters GFP-dysferlin localization. Representative images are shown for CAV3-P104L-mCherry (top) and GFP-dysferlin (bottom) in control cells (first panel) and cells expressing CAV3-P104L-mCherry (second and third panels). As previously reported, GFP-dysferlin is normally targeted to the plasma membrane and intracellular structures but retained in the Golgi of cells expressing dominant-negative, Golgi-retained CAV3 mutants (Hernández-Deviez et al., 2006). As observed for CAV3-P104L-GFP, CAV3-P104L-mCherry was localized to the Golgi and largely retained endogenous CAV3 (data not shown). Scale bar, 10 μm.

Supplementary Figure S6. Single particle tracking analysis of CD4-GFP diffusion in H9c2 cells with and without latrunculin treatment. Representative trajectories (left) and cumulative distributions (right) for D (first panel) and Range (second panel) are shown for CD4-GFP expressing H9c2 cells in control conditions (black) and after treatment with latrunculin (red). Cells were treated with 0.5 μM latrunculin B for 10 minutes prior to and during tracking experiments. The median values of D and range for CD4-GFP diffusion were ~0.03 μm^2s^-1 and ~0.3 μm (similar to other freely diffusing membrane proteins, for example see Crane and Verkman, 2008) and there was no statistically significant difference between the data sets. Data was acquired from 15-18 cells and datasets are composed of 171-224 trajectories.

Supplementary Figure S7. β-Adrenergic receptor dynamics at increasing expression levels in H9c2 cells. Transfection conditions were altered to express increasing concentrations of HA-β1- and β2AR in H9c2 cells and receptor dynamics were characterized by SPT. To quantify receptor expression levels, cells were stained with anti-HA antibody, washed, fixed and stained with secondary antibody conjugated to 605 nm Qdots. Overall β1AR and β2AR levels were calculated relative to the 50 % overexpression level calculated for the lowest levels of epitope-tagged receptor used throughout this study (1.5–fold total receptor). For both receptors, overall expression levels are presented in the same colors as cumulative distributions are plotted in. For both HA-β1- and β2AR, cells were able to effectively tether receptors up to at least ~3-fold endogenous levels. Data was acquired from 10-18 cells and represents 108-315 trajectories.
SUPPLEMENTARY MOVIE LEGENDS

Supplementary Movie 1. HA-β₁AR diffusion in H9c2 cardiomyocyte-like cells. Epitope-tagged receptors were labelled with anti-HA antibody, biotin-conjugated Fab fragments and streptavidin conjugated Qdots emitting at 655nm. Data was acquired over 20 seconds at 30 fps and is played back at 2-times acquisition rate. The images area is ~15 × 15 μm.

Supplementary Movie 2. HA-β₂AR diffusion in H9c2 cardiomyocyte-like cells. Labelling and acquisition parameters are as described for Supplementary Movie 1.

Supplementary Movie 3. HA-β₁AR diffusion in A549 alveolar epithelial cells. Labelling and acquisition parameters are as described for Supplementary Movie 1.

Supplementary Movie 4. HA-β₂AR diffusion in A549 alveolar epithelial cells. Labelling and acquisition parameters are as described for Supplementary Movie 1.

Supplementary Movie 5. HA-β₁AR diffusion in H9c2 cells expressing GFP-SAP97pdz. Labelling and acquisition parameters are as described for Supplementary Movie 1.

Supplementary Movie 6. HA-β₂AR diffusion in H9c2 cells expressing GFP-EBP50pdz. Labelling and acquisition parameters are as described for Supplementary Movie 1.

Supplementary Movie 7. Caveolar dynamics in H9c2 cells expressing low levels of CAV3-GFP. Data was acquired over 30 seconds at 10 fps and is played back at 3-times acquisition rate. The images area is ~15 × 15 μm. Fluorescence intensity decreases during data acquisition because of continuous illumination and bleaching.

Supplementary Movie 8. Caveolar appearance in H9c2 cells expressing low levels of CAV3-GFP. Data was acquired over 40 seconds at 10 fps and is played back at 6-times acquisition rate. The images area is ~3.2 × 3.2 μm.

Supplementary Movie 9. Caveolar disappearance in H9c2 cells expressing low levels of CAV3-GFP. Data was acquired over 40 seconds at 10 fps and is played back at 6-times acquisition rate. The images area is ~3.8 × 3.8 μm.

Supplementary Movie 10. Caveolar dynamics in H9c2 cells expressing high levels of CAV3-GFP. Acquisition parameters were as described for Supplementary Movie 7.
Supplementary Figure S2

Qdot 655 Fluorescence

HA–β₁AR

HA–β₂AR

Transmission

Supplementary Figure S2
\textit{\beta}_2\text{AR DyLight 649 label}

Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5

A

CAV3-P104L-GFP

CAV3-P104L-GFP+

CAV3-P104L-GFP-

CAV3

Staining

B

CAV3-P104L-GFP

CAV3

C

CAV3-P104L-GFP

HA-β2AR

GFP-Dysferlin

D

CAV3-P104L-mCherry

GFP-Dysferlin

Supplementary Figure S5
Supplementary Figure S6
Supplementary Figure S7