Novel fluorescent methods have enabled the direct visualization of the individual agonist-stimulated G-protein-coupled receptors (GPCR) trafficking in living cells. In this study the endocytic internalization of fluorescently labeled *Dictyostelium discoideum* cAMP receptors, cAR1-eYFP was visualized in real time using single-molecule fluorescent microscopy. These single-molecule experiments revealed that the fraction of cytosolic receptors increases after persistent agonist stimulation. The observed internalization process was abolished in the phosphorylation deficient mutant, cm1234-eYFP or when cells were stimulated with an antagonist Rp-cAMPS, which does not induce receptor phosphorylation. Hence receptor phosphorylation is required for internalization. This result was confirmed by confocal imaging and biochemical assays. To our knowledge, this observation illustrates for the first time phosphorylation-dependent internalization of single GPCR molecules in living cells.
4.1 Introduction

The ability of cells to communicate with and respond to their external environment is critical for their survival. The breakdown of this signaling ability is one reason for e.g. cancer, diabetes and disorders of the immune and cardiovascular systems. In the majority of signaling events initiated by extra-cellular ligands such as hormones, neurotransmitters, growth factors, the binding of these signaling molecules to seven trans-membrane G-protein-coupled receptors (GPCRs) results in the activation of G-proteins localized at the inner face of the plasma membrane (Simon et al., 1991; Clapham, 1993; Neer, 1995). In addition, agonist activation of a GPCR also results in (i) feedback regulation of G-protein coupling, (ii) receptor endocytosis and (iii) signaling through G-protein independent pathways (Ferguson, 2001). GPCR activity is the result of the coordinated balance between these inter-related processes which govern receptor signaling, desensitization and resensitization. Desensitization mechanisms have been described which regulate the activity of receptor signaling and, thereby diminish cellular responsiveness. These include alterations in the number and the affinity of surface-binding sites, and the alteration of the efficiency of receptor coupling to the G-proteins (Lohse et al., 1990; Anborgh et al., 2000; Valiquette et al., 1995; Jockers et al., 1999; Pak et al., 1999). Agonist-induced GPCR phosphorylation has been reported to be involved in receptor desensitization and internalization (Ferguson, 2001; Neer, 1995). Internalization of GPCRs after stimulation can lead to down-regulation of the number of receptors at the cell surface. However, receptor desensitization proceeds more rapidly than receptor internalization, and blocking of internalization have shown not to affect the desensitization profile (Pippig et al., 1995). Upon internalization receptors can be either recycled back to the plasma membrane (resensitization) or degraded ("down-regulated") in lysosomes. It has been suggested that the function of ligand-induced internalization of GPCRs in the desensitization and resensitization mechanisms is dependent on the type of receptor trafficking which is different for distinct subtypes of GPCRs (Ferguson, 2001). Therefore to understand the role of internalization it is necessary to unravel the mechanism of internalization in terms of quantity, how many receptors are internalized, of timescale, how fast internalization takes place, and of process, by which endocytic pathway does internalization occur. Here, these processes were investigated by single-molecule microscopy which allowed us to follow GPCR internalization one-by-one with much higher spatial-temporal resolution compared to conventional fluorescent methods. As a model we chose for the cyclic adenosine monophosphate receptor in the organism Dicyostelium discoideum.

It has been long established that the eukaryotic slime mould D. discoideum senses chemoattractants (like cAMP) by the same signal transduction mechanisms as many hormones, neurotransmitters, odorants in mammalians (Parent and Devreotes, 1996). Cloning and deletion of the four cAMP receptors (cAR1-cAR4), showed that they are essential for chemotaxis and for the developmental cycle of D. discoideum. cARs most
closely resemble chemokine receptors, which mediate chemotaxis in leukocytes (Devreotes and Zigmond, 1988). Many of the genes involved in *D. discoideum* chemotaxis are cloned, genetically disrupted and fused to the green fluorescent protein (GFP) or one of its variants (Tsien, 1989; Comer and Parent, 2002; Kimmel and Parent, 2003). However, studies under conditions of normal expression levels of the receptor have so far not been performed because of a lack of sufficient sensitivity. Previous immunofluorescence studies have shown that ligand occupancy of the primary cAMP receptor, cAR1 can lead to its clustering or internalization (Wang et al., 1988). However, more recent evidence indicated that cAR1 remained uniformly distributed on the plasma membrane even after prolonged cAMP stimulation (Caterina et al., 1995a). It was suggested that continued stimulation induced loss of ligand binding, due to a phosphorylation dependant reduction of cAR1 affinity (Xiao et al., 1997). As in other systems, the cytosolic C-terminal tail of cAR1 can be phosphorylated; however, this seems to have little impact on the overall adaptation of cAR1-mediated pathways (Caterina et al., 1995a; Caterina et al., 1995b). Cells expressing a cAR1 receptor lacking the C-terminal tail phosphorylation sites do not exhibit a ligand-mediated reduction in ligand affinity and are partially defective in the activation of the signal transducers and activators of transcription but show a normal adaptation of adenyl cyclase and guanylyl cyclase (Caterina et al., 1995a; Caterina et al., 1995b; Vaughan and Devreotes, 1988; Kim et al., 1997).

The current study investigates, in real time, the internalization of cAR1 on external stimulation by cAMP in vivo by single-molecule microscopy. Experiments were performed in *car1* cells where the endogenous cAR1 was replaced by a functional cAR1-eYFP construct (see de Keijzer et al., submitted). Here we show that cAR1 receptors were internalized upon persistent stimulation with cAMP on a timescale of $t_{1/2} = 5$ min and that this process requires receptor phosphorylation.

### 4.2 Results

#### 4.2.1 Detection of single molecules in living cells

Investigation of receptor internalization at the single molecule level in live cells was performed by focusing into the median plane of *D. discoideum car1* cells transformed with cAR1-eYFP (Fig. 4.1 A). This chimeric protein is functionally indistinguishable from wild-type cAR1 and allowed us to visualize receptors during stimulus presentation in live cells. By observation in the median plane it was possible to simultaneously image receptors located in the plasma membrane and those located in the cytosol. The position of the plasma membrane was determined from the mean of the whole image stack (Fig. 4.1 B). For analysis a membrane compartment of width of 600 nm was defined which takes into account both the optical resolution of the microscope ($0.61 \cdot \lambda/NA = 365$ nm) and any fluctuations of the membrane position during the length of the recording (5-25 s). It should
be noted that this size is larger than the actual width of the plasma membrane (5 nm). Thus, the fraction of receptors at the membrane reported in the current study was in fact overestimated by including receptors located close to the membrane. Correspondingly, the size of the internalized fraction was under-estimated. However, the error introduced by this definition was assumed to be small since receptor concentration was much higher at the membrane compared to that in the cytosol (see Fig. 4.2 A & 3 A, B). In contrast to earlier studies by confocal microscopy in which the total fluorescence of the membrane compartment was compared to that in the cytosol, the results from our single-molecule approach was less influenced by cellular autofluorescence since only signals identified as individual cAR1-eYFP were taken into account in the analysis.

**Figure 4.1: Detection of single cAR1-eYFP molecules.** A, cAR1-eYFP/carl cells were stimulated with cAMP and measured with SMM. B, the left image presents the middle plane of a typical unstimulated D. discoideum carl cell transformed with cAR1-eYFP. Individual receptors (peaks of fluorescence) were observed and located either at the membrane or in the cytosol with the position of the membrane compartment (grey line). The right image is a superposition of the mean image of a stack and that of all single-molecule positions (black dots – membrane, and grey - cytosol). The membrane compartment was defined by a width of 600 nm. This value accounts both for membrane fluctuations during recording and the optic resolution. This allows an estimation of the percentage of receptors remaining at, or near, the membrane.
4.2.2 Internalization followed in a single cell

The localization and the behavior of the receptors was followed on a single cell by recording image stacks at different time points before and while continuous stimulation with 10 μM cAMP. De novo protein synthesis was inhibited by supplementing 90 μM cycloheximide to the medium 1 h before and during the experiment. Figure 4.2 A shows the localization of the receptors in the median plane of one cell at different time points (0-15 min) before and during stimulation. The fraction of receptors staying at the membrane decreased with time, from 72% before stimulation, to 32% after 30 min of stimulation, as quantified in Figure 4.2 B. This 40% decrease measured in the median plane of the cell translates into a 60% decrease for the membrane localized receptors in the whole cell (see M&M). For an unstimulated control-cell, this fraction remained almost constant over time at ~80%, a value identical to the one found before stimulation. Those data suggested that there was a constitutive internalization of the receptors by the cells, and that this internalization was increased on persistent stimulation.

The trafficking of receptors upon internalization was further investigated, concerning recycling and/or degradation of the receptors. The total number of receptors was computed at each stimulation time point. As seen in Figure 2 C, this total number was decreased after stimulation, from N = 305 to N = 60, showing that 80% of the receptors were degraded after 30 min of cAMP stimulation. The typical time of internalization $t_{1/2} = 5$ min (Fig. 4.2 B), precedes that of degradation $t_{deg} = 13$ min (Fig. 4.2 C), as was expected for a sequential process. Little variation was observed for the unstimulated control-cells, indicating that the cAMP-induced reduction is not a photo-bleaching artifact during the measurement, but is specific to an active cell mechanism.

4.2.3 Measurement on cell populations

To further investigate this process more quantitatively and to reveal variation in the population of cells, stimulation experiments were repeated on cell populations. Cells were stimulated for a given time, washed in phosphate buffer, and subsequently maintained at 10°C to slow down de-phosphorylation kinetics. This temperature was found to be a good compromise between the maintenance of the cell metabolism and the reduction of the de-phosphorylation kinetics. It was shown that after stimulation with 10 μM cAMP, the receptors remained in the phosphorylated form for at least two hours after removal of cAMP and incubation at 4°C (Xiao et al., 1997). Therefore several cells were recorded at 10°C for no longer 30 min. Figure 4.3 A depicts two representative cells showing that in those conditions, the fraction of receptors at the plasma membrane is reduced after stimulation (from 89% to 61%). Figure 4.3 C shows the mean fraction of receptors staying at the membrane is decreased significantly by at least 20%, corresponding to 30% for the whole cell membrane, after cAMP stimulation. Hence, the single-cell findings were confirmed in population experiments which did not require observing individual cells over an extended period, thereby significantly reducing the experimental complexity.
Figure 4.2: Internalization of cAR1-eYFP followed on a single cell. Internalization before and after stimulation (10 μM cAMP) was followed on a single cell, by recording stacks of 100 images at the indicated time points. A, the top images represent the positions of all molecules in each image stack, depicting both gradual recruitment of receptors in the cytosol (grey) and global reduction of the total number of receptors over time. B, graph of the fraction of receptors remaining at the membrane versus time after stimulation. The fraction is decreased with time of stimulation. This shows that receptors were internalized after stimulation. C, graph of the total number of receptors versus time, depicting receptor degradation after stimulation, plotted in relative (left) or total (right) number. In this experiment, de novo protein synthesis was suppressed by 90 μM cycloheximide. Error bars correspond to s.e. These results are representative examples of 47 cells in 5 experiments.

The intensity distributions of the observed signals were compared in context of their localization either in the membrane or the cytosol as a result of cAMP stimulation (data not shown). The intensity distribution of the receptors located in the membrane was not affected by cAMP stimulation, indicating no cAMP induced aggregation of the receptors occurred. The intensity distribution of the receptors located in the cytosol was broadened upon 15 min of cAMP stimulation. The higher signal levels which appeared could be associated to dimers or large aggregates as described previously in Chapter 2 and indicate that there were larger aggregates of receptors in the cytosol. Photobleaching (see M&M) makes extrapolation on stoichiometry before photobleaching very unreliable since most of
the cAR1-eYFP molecules residing in multimers were bleached and the resulting monomers were observed. Therefore only comparison between the different situations was allowed and no information on the real stoichiometry was obtained.

4.2.4 Role of phosphorylation

A mutant receptor fused to eYFP, cm1234-eYFP, was used to investigate if receptor internalization is a phosphorylation-dependant process as predicted. For this mutant, all putative phosphorylation residues (18 serines) from the cytosolic C-terminus were substituted. This completely abolished cAMP-dependent phosphorylation of receptors (Hereld et al., 1994). In contrast to cAR1-eYFP (Fig. 4.3 A) the phosphorylation deficient receptor, cm1234-eYFP, was not internalized on stimulation (Fig. 4.3 B). This could be due either to a defect in plasma membrane targeting of cm1234-eYFP, or due to different kinetics of internalization and recycling. The mean fraction of receptors which stayed at the membrane was decreased significantly by at least 20% for cAR1-eYFP after stimulation, while this fraction remained almost constant for cm1234-eYFP (Fig. 4.3 C). Expression of cAR1-eYFP in wild type strains led to identical results (data not shown), indicating that the difference in genetic background (cAR1-eYFP/wt) was not causing the difference in internalization behavior. Likewise to the cm1234-eYFP, the antagonist Rp-cAMP, which binds to the cAR1-eYFP without inducing its phosphorylation (Van Haastert, 1987; Van Haastert et al., 1984) did not cause a redistribution of cAR1-eYFP between membrane-bound and cytosolic fraction (Fig 4.3 D). Taken together, those data showed that receptors were internalized upon cAMP stimulation in a phosphorylation-dependent manner.

4.2.5 Confirmation of internalization by biochemical assays

Western-blots of membrane and cytosolic fractions of receptors before and after stimulation with 10 μM cAMP were performed (Fig. 4.4). In untreated cells (0 min) the eYFP antibody detected a fusion protein of the expected size (70 kDa) mainly in the membrane fraction. Figure 4 further shows an increase in the cytosolic fraction and a decrease in membrane bound fraction after agonist treatment. cAMP stimulation in addition induced a shift of the receptor fusion protein to a form with a lower electrophoretic mobility in both membrane and cytosolic fraction. This shift was also observed for wild-type cAR1 and is known to be due to phosphorylation of cAR1 on either Ser-303 or 304 (Caterina et al., 1995a; Hereld et al., 1994). The fact that only ~ 50% of the cAR1-eYFP was upshifted after 15 minutes incubation with 10 μM cAMP, a condition that would result in over 95% upshift of wild-type cAR1 (Vaughan and Devreotes, 1988), suggests that the C-terminal eYFP moiety either decreases the kinetics of phosphorylation (or increases dephosphorylation), or renders a fraction of the receptor inaccessible to the kinase responsible for phosphorylation. Post-lysis dephosphorylation could also explain this observation. The cytosolic fraction resembled that of the internalized receptors, which
remained stably phosphorylated after global stimulation. This indicates that the cAR1-eYFP fusion protein was internalized upon stimulation, and that its phosphorylation state was unchanged when internalized.

**Figure 4.3:** Phosphorylation does affect receptor localization at the membrane. A,B typical cells measured at 10°C, expressing either cAR1-eYFP (A) or cm1234-eYFP (B) before or after 15 min stimulation with 10 μM cAMP. The fraction of molecules in the cytoplasm is increased after 15 min of stimulation for cAR1-eYFP, but not for the phosphorylation deficient mutant cm1234-eYFP. C, graph of the fraction remaining at the membrane versus time after cAMP stimulation, normalized at 0 min for comparison. This fraction significantly decreased in time for cAR1-eYFP, but not for cm1234-eYFP. D, the fraction remaining at the membrane significantly decreased in time for cAR1-eYFP when stimulated by cAMP, but not when stimulated by the antagonist Rp-cAMPS, which binds to the receptors without inducing their phosphorylation. Error bars correspond to s.e.m.

**Figure 4.4:** Gel mobility shift assay. Western-blots of membrane (m) and cytosolic (c) fractions of cAR1-eYFP, before and 15 or 30 min after stimulation with cAMP.
4.2.6 In vitro imaging after in vivo stimulation

To confirm that receptors were internalized upon persistent stimulation, an independent method established by others was used. cAR1-eYFP/car1 and cm1234-eYFP/wt cells were stimulated for different time periods with 10 μM cAMP and fixed immediately after stimulation in methanol at -20°C. Subsequently the fixed cells were imaged in a confocal microscope. Figure 4.5 A presents typical cells imaged.

Figure 4.5: In vitro imaging of receptors localization. A, cells expressing cAR1-eYFP or cm1234-eYFP were stimulated in vivo for different length of time and fixed with methanol at -20°C and imaged in a confocal microscope. Cells expressing cAR1-eYFP display mainly a membrane staining before stimulation, and a gradual appearance of cytosolic fluorescence following stimulation. Cells expressing cm1234-eYFP always display both membrane and cytosolic fluorescence, without any change with stimulation. Those images thus confirm the in vivo result that stimulation induces internalization of cAR1, but does not affect internalization of the phosphorylation deficient mutant cm1234. B, quantification of the membrane fraction, computed as the mean fluorescence at the membrane relative to the mean fluorescence of the cell and normalized at 0 min. This graph shows that the membrane fraction is decreasing for cAR1, while remaining almost constant for cm1234. Error bars correspond to s.e.m.

To quantify the level of membrane-located receptors, cells were detected by thresholding and the mean fluorescence signal was computed at the membrane and compared to that in the cytosol. This lead to a membrane fraction, similar to the one computed in the single-molecule measurements. As can be seen in Figure 4.5 B, the in vitro measurements confirmed the in vivo results in that cAMP-stimulation induced
internalization of cAR1. Membrane localization was gradually re-localized into the cytosol after stimulation. Likewise, as shown in the images and in the figure (Fig. 4.5 A&B), stimulation did not trigger internalization of the phosphorylation deficient mutant cm1234.

4.3 Discussion

G-protein coupled receptors comprise the single largest gene family with more than 1000 genes in the human genome. These receptors mediate responsiveness to numerous stimuli including hormones, neurotransmitters, peptides, odorants and light. Since they are the most common targets of therapeutic drugs it is appealing to study their molecular behavior during stimulus presentation. Although the endocytic membrane trafficking plays multiple roles in GPCR signaling, the relationship between spatial distribution of receptors and their function are largely unknown. Internalized GPCRs can follow two different pathways: they can be either recycled back to the membrane or targeted to lysosomes for their degradation (Fig. 4.6). Recently tagging of GPCRs with the green-fluorescent protein has enabled the direct visualization of real-time trafficking of GCPRs in living cells.

In the current study, for the first time, single-molecule imaging techniques with superior lateral resolution as compared to regular fluorescence microscopy was successfully used to monitor internalization of G-protein coupled receptors molecule-by-molecule in live cells. We developed this method for the G-protein coupled cAMP receptor, cAR1-eYFP in Dictyostelium discoideum. The results showed that our methodology can be viewed as an universal tool for various types of GPCRs in all cell types. Using single-molecule fluorescence microscopy and biochemistry, we have shown that the cAR1-eYFP was partly internalized and degraded after persistent stimulation. As shown by the use of a phosphorylation deficient mutant and by an antagonist that does not induce receptor phosphorylation, this process requires phosphorylation of the receptors to occur. Preliminary experiments further showed that receptors were still internalized when actin filaments were disrupted by treatment with cytochalasin (data not shown).

Before, data about internalization of cARs upon stimulation in the literature were conflicting. Some studies, based on biochemistry (Padh and Tanjore, 1995; Van Haastert et al., 1992) and on immuno-cytochemistry (Wang et al., 1988) reported internalization, while others stated that receptors stayed at the membrane, although dealing with cells in suspension (Caterina et al., 1995b) or shorter stimulation times (Xiao et al., 1997). Nevertheless, our results clearly show that the internalized fraction, as measured with the highly sensitive single-molecule technique, is rather low ~30% and may thus not be detected by classical methods. The receptors were internalized on a timescale of t1/2 = 5 min. Desensitization of the chemotactic response is a poorly understood, integral process of different mechanisms like adaptation, sequestration and down-regulation. Adaptation is the rapid and reversible attenuation of responses (30s) and is phosphorylation independent. Although a profound loss of adaptation to the chemoattractant signal was seen in cells lacking the PI3 phosphatase PTEN, the response eventually subsided after a few minutes.
This corresponds with our timescale and could indicate that the ligand-induced internalization is another desensitization mechanism. Our data further established that the major part (80%) of the receptors was degraded after internalization. However, this does not exclude a possible recycling of a sub-fraction of the receptors at the same time, as suggested previously (Padh and Tanjore, 1995).

The sensitivity of single-molecule microscopy allowed the detection of receptor internalization in live cells. This process is likely to be involved in receptor desensitization and resensitization. It was suggested that internalization is a component of the cell physiological response during *D. discoideum* developmental cycle, including the phases of chemotaxis and aggregation. Further questions remain open, as depicted by question marks in Figure 6. It will be important to determine the endocytic pathway followed by the receptors (e.g. clathrin-mediated or caveolin-mediated endocytosis (Tsao and von Zastrow, 2001)), whether the ligand is internalized together with the receptor, what the dynamics of G-proteins during this internalization process is, whether G-proteins are dissociated from internalized receptors, and if internalized receptors are still able to signal through other pathways (as reported for the β-adrenergic receptor and MAPK (von Zastrow, 2001)). In the near future use of even advanced single-molecule imaging with multi-color tagging of proteins and ligands will enable us to tackle those questions in detail.

**Figure 4.6: Internalization of stimulated receptors.** Summary of the events occurring during stimulation, phosphorylation and internalization of cAR1. Remaining questions about the endocytic pathway, the phosphorylation state of internalized receptors and the localization of the G-protein and the ligand, are marked.
4.4 References


4.5 Materials and methods

4.5.1 cAR1-eYFP fusion protein

pYU21 plasmid (constructed by Yu Long, Peter Devreotes laboratory), a plasmid of 14.10 Kb, contains eYFP fused to the C-terminus of cAR1. This plasmid was expressed in car1- cells by electroporation. Clones were grown up in a Petri dish in HL5-medium containing 10 μg/ml G418. For expression of phosphorylation-deficient cAR1 tagged with eYFP (cm1234-eYFP) the C-terminal cytoplasmic domain of the site-directed mutant cm1234 was amplified by PCR using primers CACCTATTTGAGTGTATCCC & GGCTAGCTGGTGGATTATTTCCTTGACCATTTG and subcloned in place of the corresponding wild-type sequence in pYU21 by replacement of a BstXI-NheI fragment. Cell-lines were maintained in 6-well plates in axenic medium with addition of penicillin and streptomycin at 22°C. Cells were maintained overnight in low fluorescence medium (Liu et al., 2002) and starved for 4-6 hours in phosphate buffer prior to measurement. All measurements were performed at 22°C.

4.5.2 Immunobloting

cAR1-eYFP/car1- cells were developed in 10 mM phosphate buffer (PB), pH 6.5, at a density of 107 cells/ml for 6h. First, cells were pre-incubated with 5 mM caffeine for 30 min and stimulated for different time periods with 10 μM cAMP and 10 mM DTT. After centrifugation at 1500 g for 2 min cells were re-suspended to 2×108 cells/ml in cold PB supplemented with a protease inhibitor cocktail (Complete TM Mini EDTA-free, Roche). The cells were lysed through a nuclear pore filter with a pore size of 3 μm. The lysate was centrifuged at 10000 g for 2 min. A sample of the supernatant was mixed with SDS-buffer to a density of 108 cells equivalent/ml. The pellet was washed with PB and solubilized with SDS-buffer to a density of 108 cells equivalent/ml. The supernatants and membrane protein fractions were resolved by SDS-page on 10% gels along with a set of protein MW standards. cAR1-eYFP was detected by immunoblot with anti-GFP antibody (Clontech, Palo Alto).

4.5.3 Single-molecule microscopy

The experimental set-up for single molecule imaging has been described in detail previously (Harms et al., 2001). The samples were mounted onto an inverted microscope (Zeiss) equipped with a 100x objective (NA=1.4, Zeiss). A region-of-interest was set to 50x50 pixel at a pixel-size of 220 nm. Measurements were done by illumination of the samples for 3 ms at 514 nm (Ar+ laser, Newport Spectra Physics) at intensity of 1 kW/cm². The cells were photo-bleached for a period of 2.5-5 s at an intensity of 1 kW/cm² prior to measurement. Use of appropriate filter combinations (DCLP 530, HQ570/80, Chroma Technology and OG 530, Schott) permitted the detection of the fluorescence signal by a
liquid nitrogen-cooled CCD-camera (Princeton Instrument). The total detection efficiency of the experimental setup is 8%. A stack of 100 images were recorded with a timelag between the images of $t_{lag} = 55$ ms. Images were analyzed using programs written in Matlab (Schmidt et al., 1995, de Keijzer et al, submitted).

After individual receptors were identified the membrane vs the cytosolic fraction was easily determined by counting the signals in the median plane. For extrapolation of those results to the whole cell the median fraction has to be multiplied by 3/2. Assuming that receptor localization is not polarized but homogeneous over the whole cell (as reported before (Wang et al., 1988; Xiao et al., 1997)), the ratio of receptors located at the membrane in the median plane is proportional to the total ratio for the whole cell. If we assume a spherical shape for the cell, with radius $r$, the volume is $4/3 \pi r^3$, and the surface is $4\pi r^2$. Thus, the ratio of membrane to volume is $3/r$. In the median plane, which we assume as cylinder, of height $h \approx 1$ µm corresponding to the depth of focus, the volume is $\pi r^2 h$ and the surface $2\pi rh$, leading to a ratio membrane to volume of $2/r$. Therefore, the results found in the median plane as plotted in the Figures 4.2 & 4.3 under-represent plasma membrane-localized receptors and must be corrected by a factor of 3/2.