A FRET map of membrane anchors suggests distinct microdomains of heterotrimeric G proteins

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Summary
The standard model of heterotrimeric G protein signaling postulates a dissociation of Ga and Gβγ subunits after activation. We hypothesized that the different combination of lipid-modifications on Ga and Gαβγ subunits directs them into different microdomains. By characterizing rapidly and at high sensitivity 38 fluorescence resonance energy transfer (FRET) pairs of heterotrimeric-G-protein constructs, we defined their microdomains in relation to each other, free from the constraints of the raft/non-raft dualism. We estimated that in a cell ~30% of these membrane-anchored proteins are mostly clustered in 3400-16,200 copies of 30-nm microdomains. We found that the membrane anchors of Ga and Gαβγ subunits of both the Gβi/o and Gq family co-cluster differently with microdomain markers. Moreover, anchors of the Gαq/i/o and Gαq subunits co-clustered only weakly, whereas constructs that contained the anchors of the corresponding heterotrimers co-clustered considerably, suggesting the existence of at least three types of microdomain. Finally, FRET experiments with full-length heterotrimeric G proteins confirmed that the inactive, heterotrimerized Ga subunit is in microdomains shared by heterotrimers from different subclasses, from where it displaces upon activation into a membrane-anchor- and subclass-specific microdomain.

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Key words: FRET, Heterotrimeric G protein, Microdomain, Nanodomain, Raft

Introduction
G-protein-coupled receptors (GPCRs) form one of the largest and pharmacologically most important receptor families (Fredriksson et al., 2003). In the canonical signaling pathway, they activate heterotrimeric G proteins, which then dissociate and allow Gα and Gβγ subunits to specifically access effectors on the intracellular side of the plasma membrane (Gilman, 1987). How this specificity is achieved is only partially understood (Hamm, 1998).

Data on G proteins of the Ras-family suggest that differential partitioning into microdomains could explain effector specificity (Voice et al., 1999; Yan et al., 1998). Ras proteins are structurally most divergent at their C-termini, which contain different membrane-anchoring motifs (Prior and Hancock, 2001). This region directs an isoform-specific and, for H-ras and N-ras, also activation-state-specific lateral segregation in microdomains of the plasma membrane (Prior et al., 2003; Roblat et al., 2004; Roy et al., 2005), which suggests that their microlocalization contributes to specific effector activation. These Ras microdomains have diameters in the range of nanometers and may depend on cholesterol. Additional evidence for microdomains on the outer and inner leaflet of the plasma membrane of living cells has been provided using FRET (Meyer et al., 2006; Sharma et al., 2004; Zacharias et al., 2002), which allows to study molecular interactions and assemblies in the nanometer range in living cells.

The lateral organization of heterotrimeric G proteins, however, is less well characterized. Immunofluorescence data on heterotrimeric G proteins have shown a subclass-specific colocalization with caveolin and raft markers (Oh and Schnitzer, 2001). Biochemical experiments furthermore revealed different fractionation patterns of active and inactive transducin, the heterotrimeric G protein of photoreception, in detergent-resistant membrane preparations (Nair et al., 2002; Seno et al., 2001). These data suggested that subclass and activation state also influence the membrane organization of heterotrimeric G proteins. However, the correlation of these data with plasma membrane lipid rafts, which were proposed as submicroscopic cholesterol- and sphingolipid-rich dynamic lipid-protein structures in living cells (Simons and Ikonen, 1997), is questionable (Lichtenberg et al., 2005).

Here, we investigated the activation state and subclass dependence of the microdomain localization of heterotrimeric G proteins in living cells. We acquired highly reproducible FRET data on a cytometer, which was much faster than the common microscopy-based approaches. Thus, we were able to analyse a high number of FRET pairs, which allowed us to characterize the overlap of their microdomains by the magnitude of their FRET. Our results show that membrane anchors of Gα and Gαβγ subunits fused to fluorescent proteins cluster differently with distinct microdomain markers. Moreover, fluorescent membrane anchor constructs derived from heterotrimers of the Gβi/o and Gq subclass cluster together, whereas anchor constructs of the Gαq/i/o and Gαq subunits do not lead to considerable co-clustering. In addition, we...
determined activation-induced FRET changes of fluorescently
tagged full-length heterotrimeric G proteins, which
corresponded to those predicted by our clustering data of the
membrane anchors. Our data therefore suggest that
heterotrimeric G proteins of the G_{i/o} and G_q subclass show a
membrane-anchor and activation-state-specific microdomain
localization, which may allow these G proteins to specifically
and efficiently access effectors.

Results

G-protein-derived membrane anchors direct partial
clustering in microdomains of the inner leaflet of the
plasma membrane

The submicroscopic clustering of appropriately labelled
proteins in microdomains can be studied by analysing the
dependence of FRET on the expression level of these proteins
(Meyer et al., 2006). Here, by using a calibrated flow
cytometer, we analysed rapidly and at high sensitivity 38
monomeric cyan fluorescent protein (mCFP) and monomeric
yellow fluorescent protein (mCit) FRET pairs, which were
either heterotrimeric-G-protein-derived constructs or
constructs serving as microdomain markers (Fig. 1A).

HEK293 cells were transiently co-transfected and
fluorescence of at least 10^5 cells was measured. Fluorescence
intensity of acceptors and donors covered four orders of
magnitude, corresponding to the entire range of observable
protein expression. We calculated FRET per cell, using an
adapted sensitized acceptor-emission method (Gordon et al.,
1998). At constant donor-acceptor ratio of 1:1, FRET
efficiencies increased with increasing acceptor concentrations
towards a maximal value, E_{max} (Fig. 1D), which we determined
as a free fit parameter in equation 1 (see Materials and
Methods). Randomly distributed fluorophores in the bulk
membrane would amount to less than 5-10% FRET (Fig. 1D
left, dashed line) (Wolber and Hudson, 1979) at concentrations
where we often observed E_{max} \approx 30\% (Fig. 1E, Fig. 4C, Fig.
5A), indicating clustering in microdomains. Supposing that
E_{max} is reached at the most dense cubic packing of the

![Diagram](image.png)

**Fig. 1.** Analysis of FRET on the cell membrane demonstrates the distinct levels of co-clustering of microdomain markers. (A) Illustration of our
reductionistic approach to study activation-state-dependent microdomain localization of heterotrimeric G proteins. (Top) Activation followed by
dissociation of the heterotrimer leads to a separation of \( G_\alpha \) and \( G_{\beta\gamma} \) subunits. (Bottom) Only the lipid anchors of inactive heterotrimer and
active \( G_\alpha \) were fused to fluorescent proteins, and the microdomain localization of these G-protein-anchor constructs was studied in relation to
microdomain markers (see B and C) using FRET between mCFP as a donor and mCit as an acceptor. (B) Schematic representation of
microdomain markers with their lipid anchors. P, palmitoyl; G, geranylgeranyl; F, farnesyl; pb, polybasic sequence. Source refers to the proteins
from which targeting sequences were derived. (C) Subcellular localization of microdomain markers imaged by confocal microscopy. All
constructs were predominantly localized to the plasma membrane, with minor labeling of internal membranes or, in the case of the Rac1-
derived construct, nuclear labeling. Only the mCit constructs are representatively shown. Bars, 10 \( \mu \)m. (D) By plotting the FRET efficiency (E)
against the normalized acceptor surface concentration (cA) at a constant donor mole fraction (x_D=0.50±0.17), we obtained information about
the clustering of donor and acceptor fluorophores. A random distribution of fluorophores (Wolber and Hudson, 1979) cannot describe our data
(left, hashed curve). We found that the FRET efficiencies increased after a cA offset towards a plateau value E_{max} (indicated by solid horizontal
line in the left plot). We therefore adapted the double exponential function of Wolber and Hudson (Wolber and Hudson, 1979), further taking
the cA offset and the maximum efficiency E_{max} into account. This lead to fits which described all of our FRET data adequately (from left to
right, \( \chi^2 \): 22.6, 25.3 and 9.7). Each datapoint was calculated on a single cell. Representative examples of indicated FRET pairs are shown.
(E) The E_{max} matrix of microdomain-marker FRET pairs. FRET values of mCFP-tH/mCit-tH and mCFP-tK/mCit-tK are significantly higher
(bold) than those of tH-polybasic pairs (\( P<0.001 \) or \( P<0.05 \), respectively, 2-tailed Student’s t-test). No significant differences were found for
consistently high mCFP-polybasic/mCit-polybasic sequence pairs (bold). E_{max} values are given in percent ± s.d.; n, number of independent
experiments.
constructs (modelled as cylinders with a diameter of 3.4 nm), a local concentration of 87,000 fluorophores/μm² was estimated. However, E_max was already reached at 200-500 acceptor molecules/μm² (Fig. 1D left; supplementary material Fig. S1A), implying that fluorophores would be concentrated 87 to 218 times in such microdomains. We can furthermore assume that E_max is composed of FRET from microdomains and the bulk of the membrane. Thus, we can calculate the fraction of fluorophores in microdomains (supplementary material Fig. S1B), using equation 2 (see Materials and Methods). At a typical value of E_max=30% (Fig. 1E, Fig. 4C, Fig. 5A) approximately 26-34% of the membrane-anchored fluorophores were estimated to be in microdomains. This is in very good agreement with FRET data of GPI-anchored proteins on the exoleaflet (Sharma et al., 2004) and electron microscopy (EM) data from the minimal membrane anchor of H-ras fused to GFP on the inner leaflet of the plasma membrane (Plowman et al., 2005), which were both in a minor fraction of 20-40% in nanoclusters. Moreover, using a typical domain size of 30 nm diameter (supplementary material Fig. S1C) and the cell surface divided by the above concentration factors as an estimate for the total microdomain area, we can calculate 3400 to 16,200 microdomains per cell (or 6.5-16/μm²).

FRET characterizes the lateral segregation of microdomain markers
The dual-palmitoyl–farnesyl membrane anchor of H-Ras was shown to cluster cholesterol dependently in nanometer-sized microdomains, whereas the polybasic sequence of the farnesyl membrane anchor of K-ras 4B (hereafter K-ras) clustered cholesterol independently (Plowman et al., 2005; Prior et al., 2003). Moreover, these membrane anchors, abbreviated tH and tK, for tail of H-Ras and K-ras 4B, respectively, clustered in non-overlapping microdomains (Prior et al., 2003). We therefore fused them to mCFP or mCit in order to generate microdomain markers for different microdomains (Fig. 1B,C). To expand the range of membrane anchors, the targeting sequence of Rac-1 (hereafter abbreviated tR) was chosen to construct another microdomain marker (Fig. 1B,C). This marker contains a polybasic sequence similar to that of K-ras and a geranylgeranyl moiety. Because of the common polybasic sequence, we expected it to also have similar lateral segregation properties as the K-ras-derived marker. We found high E_max values above 25% FRET efficiency for mCFP-tR/mCit-tR and mCFP-polybasic/mCit-polybasic FRET pairs (Fig. 1E, bold), consistent with the nanoclustering found by EM analysis (Plowman et al., 2005; Prior et al., 2003). However, E_max values of the tH/polybasic pairs were significantly smaller (Fig. 1E, non-bold values), but still above the 5-10% of randomly distributed fluorophores, suggesting some co-clustering in HEK293 cells. Consistent with the negligible FRET from soluble fluorophores at these concentrations (supplementary material Fig. S2E), FRET from the nucleus is close to zero for mCFP-tR/mCit-tR. Bars, 5 μm.

![Fig. 2. Sensitized acceptor emission FRET imaging confirms the distinct FRET levels on the plasma membrane. Left to right: FRET-channel images, FRET-efficiency images and dependencies of the FRET efficiency of indicated FRET pairs on the acceptor intensity, corresponding to approximately 500-2000 acceptors/μm². Each datapoint was calculated on one region of interest on plasma membranes with donor mole fractions of xD=0.5±0.17 were analysed. Note that for mCFP-tH/mCit-tH the FRET originating from putative internal membranes show similar values as that originating from the plasma membrane. Consistent with the negligible FRET from soluble fluorophores at these concentrations (supplementary material Fig. S2E), FRET from the nucleus is close to zero for mCFP-tR/mCit-tR. Bars, 5 μm.](image-url)
In addition, we developed a quantitative FRET assay for FRET from internal membranes, to determine the FRET contribution from internal membranes that were also populated by our constructs, albeit to a minor extent (e.g. Fig. 1C). We co-expressed each of our constructs with a geranylgeranylated FRET partner (supplementary material Fig. S2B), which binds to all major internal membranes (Goodwin et al., 2005; Rocks et al., 2005; Silvius et al., 2006), and analysed them in the same way as the pairs of the microdomain marker. This assay revealed that, at high expression levels of acceptor, less than 10% FRET efficiency was reached (supplementary material Fig. S2C,D), which is also consistent with our FRET-imaging data. In addition, any contribution from soluble fluorophores in the cytoplasm or nucleoplasm was negligible (supplementary material Fig. S2E).

Membrane-targeting sequences of G\textsubscript{\textalpha} and the G\textsubscript{\textalpha}/G\textsubscript{\beta}\gamma heterotrimer direct localization to different microdomains

The standard model of heterotrimeric G protein signaling postulates a dissociation of G\textsubscript{\textalpha} and G\beta\gamma subunits after receptor activation. We hypothesized that heterotrimer, G\alpha and G\beta\gamma subunits localize to different microdomains because of their different lipid modifications.

In order to describe the microdomain localization of heterotrimer and G\alpha subunit, we started with a reductionistic approach (Fig. 1A). We fused the membrane-anchoring sequences of active G\textsubscript{\textalpha} (N\textsubscript{\textbeta}2) or inactive G\textsubscript{\textalpha}\beta\gamma (N\textsubscript{\textbeta}2-C\textgamma) subunits to fluorescent proteins (Fig. 3A). These constructs localized predominantly to the plasma membrane (Fig. 3B) and allowed us to determine their microlocalization in relation to the fluorescent microdomain markers by whole-cell fluorescence measurements.

Next, we characterized the microlocalization of G\textsubscript{\textalpha} membrane anchors. Comparison of the \textit{E}_{\text{max}} values of the FRET pairs of N\textsubscript{\textbeta}2-mCFP and microdomain marker (or -mCit) revealed a characteristic set of \textit{E}_{\text{max}} values for the G\textsubscript{\textalpha} anchor, with higher FRET values for markers that contain polybasic sequences, than for tH (Fig. 3C first column). The \textit{E}_{\text{max}} values, again, reflect the differential co-clustering of the microdomain overlap of microdomain markers and the heterotrimeric-G-protein-derived membrane anchor constructs. We called the set of \textit{E}_{\text{max}} values with the microdomain markers a FRET vector (e.g. any column in Fig. 3C), as it defines the position of the heterotrimeric-G-protein-derived construct in a coordinate system defined by the three microdomain markers.

The FRET vectors of the G\textsubscript{\textalpha} derived anchor construct (Fig. 3A,C first and third column) was mainly associated with the lipid modifications, as we verified by exchanging the membrane-targeting sequence of G\textsubscript{\textalpha} with that of Lyn-kinase, which also directs a myristoylation and palmitoylation (Fig. 3A,C second column). Comparison of FRET vectors of G\textsubscript{\textalpha} anchor constructs (Fig. 3A,C third column) with those of G\textsubscript{\textalpha}\beta\gamma constructs (Fig. 3A,C fourth column) revealed that the FRET vectors are different. The \textit{E}_{\text{max}} value of N\textsubscript{\textbeta}2-C\textgamma-mCFP-tH is significantly larger, and the \textit{E}_{\text{max}} value of N\textsubscript{\textbeta}2-C\textgamma-mCit-mCFP-tR is significantly smaller, than the corresponding G\textsubscript{\textalpha}\gamma anchor construct values (Fig. 3C). Again, FRET imaging confirmed these relations and the FRET levels on the plasma membrane (supplementary material Fig. S2A). In conclusion, the different FRET vectors of G\textsubscript{\textalpha} and cognate anchor constructs of heterotrimeric G proteins imply that the G\textsubscript{\textalpha} subunit has a different microlocation after activation/dissociation, if the lipid anchors predominantly determine the microdomain localization.
Lipid anchors of Goq and Gαqβγ also localize to different microdomains, suggesting that Goq and Gαqβγ generally localize to different microlocations.

In the next step, we tested, whether these conclusions also apply for the membrane anchors of the Gq subclass of heterotrimeric G proteins. An analogous set of constructs was created using the membrane-targeting sequence of Goq that becomes dually palmitoylated in the native protein. Unfortunately, this sequence alone is not sufficient to confer membrane localization of the protein, which remains in the cytoplasm (Evanko et al., 2000) (and data not shown). However, our results with the Goqα+β and Lyn-kinase-derived constructs suggested that the microlocalization is primarily governed by the lipid anchors. Consequently, we used the similarly dually palmitoylated sequence from GAP-43 (NGAP-43), which was sufficient for plasma membrane trafficking (Fig. 4A,B), as a surrogate. The FRET vector of this construct (NGAP-43-mCFP) was again markedly different from the one of the corresponding heterotrimer construct, NGAP-43-Cy-mCFP (Fig. 4D,C compare column 1 with 2).

We further validated the use of the membrane anchor of GAP-43, by creating a heterotrimer construct that combined the Goq N-terminal sequence with the Gy2-derived C-terminal geranylgeranylation site. Consistent with data on plasma membrane trafficking of heterotrimeric G protein (Michaelson et al., 2002), which also suggest two plasma-membrane-targeting signals, the resulting NqCy-mCFP localized predominantly to the plasma membrane (Fig. 4A). Whereas the FRET vector of this construct was very similar to the one of NGAP-43-Cy-mCFP, the Emax of NqCy-mCit/mCFP-tR was ~50% smaller than the Emax of NGAP-43-Cy-mCFP/mCFP-tR (Fig. 4C, last two columns).

We propose that the extended stretch of basic residues on one side of the α-helix of the Goq-derived peptide is responsible for this difference (Fig. 4E). However, the Emax of the two Goqα+β anchor constructs (NGAP-43-Cy-mCFP/NqCy-mCit; Fig. 5A, dark grey area on the right) was as high as the one of mCFP-tH/mCit-tH or mCFP-tK/mCit-tK (Fig. 1E), indicating that they, nevertheless, considerably co-microlocalize. In summary, these data suggest that Goq localizes to a different microdomain than its heterotrimer. Again, lipid anchors predominantly determine their microlocalization, which can be modulated by the surrounding amino acids that may contact the membrane. Taking also into account our results with the Goqα anchor construct, we conclude that monomeric Goq protein and the heterotrimer generally localize to different microdomains.

FRET data of membrane anchors suggest that Goqαβγ and Gαqβγ co-microlocalize considerably, whereas Goqα+β and Goq do not.

In addition to the anchor constructs of the Gq heterotrimer, we compared directly the microdomain overlap of the anchor constructs of the Goq and Goqα heterotrimers. Consistent with the
**Fig. 5.** (A) The direct comparison of G-protein-anchor FRET pairs suggests that GoiG and GoiOβγ share the same microdomain, whereas GoiO and GoiQ do not. The Emax values are given in % ± s.d.; n, number of independent experiments. Emax values of heterotrimeric construct FRET pairs are not significantly different (dark grey), whereas the values of Go construct FRET pairs Ni2-mCFP/Ni2-mCit and NGAP-43-mCFP/Ni2-mCit (light grey) are significantly different (P<0.05, 2-tailed Student’s t-test). Blue and yellow column headings highlight mCFP- and mCit-labeled constructs, respectively. (B) The FRET map of heterotrimeric G-protein microdomains based on the results of their membrane anchors. This scheme is based on the Emax relationships, where a high Emax value relates to a large overlap of the microdomains or a higher probability of the respective molecules to co-cluster. As an example, the arrow shows how the GoiO subunit displaces after activation. Its starting microdomain has a lesser ‘proximity’ to the tR–microdomain-marker (low FRET), than the destination microdomain of active GoiO (high FRET). Thus, activation results in an increase of FRET for the FRET pair GoiI0-construct/tR (Fig. 6C).

Very similar FRET vectors of the heterotrimer anchor constructs that indicated similar lateral segregation (Fig. 3C, Fig. 4C), we found a high Emax for NGAP-43-Cy/mCFP/Ni2-Cy-mCit (Fig. 5A, dark grey area on the right). Therefore, Gq and Goi heterotrimers may also co-cluster considerably. By contrast, comparison of the FRET values of Ni2-mCFP/Ni2-mCit and Ni2-mCFP/Ni2-mCit, suggested that Gq and Goi do not extensively co-cluster (Fig. 5A, light grey area in the middle), as already suggested by their different FRET vectors (Fig. 3C, Fig. 4C).

How do membrane anchors of Ga and Gαβγ subunits from different subclasses microlocalize relative to each other? We generally found low Emax values also between Ga- and Gαβγ-anchors from different subclasses (Fig. 5A, white area), suggesting little co-clustering, similar to the FRET pairs of tH/tR or tH/tK (Fig. 1C). An exception is the comparatively high FRET of Ni2-mCFP/Ni2-Cy-mCit (Fig. 5A, white area on the left). However, the analogous Ni2-mCit/NGAP-43-Cy-mCFP had a significantly smaller FRET value (Fig. 5A), making it hard to draw a definite conclusion on the co-microlocalization of GoiOγ- and GoiOβγ-derived membrane anchor constructs. We conclude that Ga subunits from the GoiQ and GoiO subclases localize to different microdomains, while the Gαβγ heterotrimers localize to microdomains that considerably overlap (Fig. 5B).

Predictable FRET changes of full-length heterotrimeric G proteins after activation confirm their relocalization to different microdomains

So far we have based our conclusions on the microlocalization of heterotrimeric G proteins on FRET between fluorescent proteins targeted with G-protein-derived membrane anchors. This was a necessary reduction for the construction of our FRET map of heterotrimeric G protein membrane anchors (Fig. 5B), which required that the distance of the fluorescent proteins to the inner leaflet is very similar for all constructs. We next tested for activation-induced microdomain relocalization of full-length Ga proteins, which can be deduced from our Emax relations (Fig. 3C, Fig. 4C) and can be followed in our FRET map (Fig. 5B). We used a fluorescent protein labelling strategy that has been shown to produce functional heterotrimeric Gα subunits (Leaney et al., 2002). Fluorescent proteins were fused to the N-terminus of GoiQ2 and GoiQ, and targeted using the previously employed targeting sequences (Fig. 6A,B). FRET between microdomain markers and full-length G-protein constructs was considerably smaller than for the membrane-anchor-only FRET pairs, probably reflecting higher sterical requirements of the full-length G protein. We therefore decided to use a spectrofluorometer of high sensitivity to detect FRET changes after stimulating cells with the membrane-permeating AlF4−, which induces a conformational change in heterotrimeric G proteins that corresponds to the transition state of GTP hydrolysis, resulting in dissociation of Ga from the Gβγ subunit (Sondek et al., 1994). After stimulation of cells with AlF4−, we found a relative decrease in FRET for NGAP-43-mCFP-GaQ/mCit-tH and NGAP-43-mCFP-GaO/mCit-tK, and no change for NGAP-43-mCFP-GaO/mCit-tR (Fig. 6C). However, FRET increased for Ni2-mCit-GaQ2/mCit-tR and Ni2-mCFP-GaO/mCit-tR. Intriguingly, all of these FRET changes corresponded to the changes expected from the Emax relations of the corresponding FRET pairs of the Ga- and heterotrimer-anchor constructs (Fig. 3C, Fig. 4C). Control experiments with the corresponding anchor constructs, showed only small FRET decreases (NGAP-43-mCFP/mCit-tH=−8±1, n=3; NGAP-43-mCFP/mCit-tR=−5, n=1; Ni2-mCit/mCFP-tR=−7±4, n=2), which we explain with the rearrangements of endogenous, unlabelled heterotrimeric G proteins in microdomains. In summary, our data of anchor constructs allowed us to predict the FRET changes of the full-length Gα constructs. Moreover, their microdomain relocalization was determined by the membrane anchor and not the subclass of the G protein.

**Discussion**

Our FRET data on fluorescently tagged heterotrimeric-G-protein-derived membrane anchors and full-length proteins suggest a membrane-anchor-specific lateral segregation of heterotrimeric G proteins in microdomains.

We used a cytometer approach to rapidly characterize our FRET pairs on a whole-cell basis. Nevertheless, the sensitized acceptor-emission FRET efficiencies calculated on plasma membrane signals were in the same range as those obtained by
our cytometer approach. Both our cytometer FRET and imaging FRET show more scatter of the data points than a similar FRET-imaging approach, where clustering of a GPCR in microdomains was studied (Meyer et al., 2006). This may be explained by our use of fluorescent proteins, because in the other study a novel labeling method was exploited that allowed precise, exogenous control of the mole fraction of the labelled donor, and facilitated also calibration for the surface concentration. Nevertheless, some variability of their data can be seen, suggesting that cell-to-cell variations account for it. However, the cytometer allowed to observe the entire range of protein expression on a single, continuous scale; this revealed the characteristic trends that allowed highly reproducible fitting.

The good reproducibility of our cytometer-FRET approach has recently been demonstrated because the $E_{\text{max}}$ values and $E_{\text{max}}$ relations could largely be reproduced in BHK cells and using a different cytometer (D.A., unpublished data). Moreover, a surprisingly high sensitivity could be shown by its ability to detect the influence of individual amino acid exchanges on an 8-dimensional (8 different microdomain marker) lateral segregation FRET vector of H-ras, stressing the potential of this approach (D.A., unpublished data).

The assumptions that allowed us to derive the fraction of our anchor constructs in microdomains are simplistic, but produce numbers that are in striking agreement with those obtained with different methods (Plowman et al., 2005; Sharma et al., 2004). The actual packing of the fluorophores at concentrations where $E_{\text{max}}$ was determined may not be the most dense packing. Therefore, the actual FRET efficiency within the microdomain/nanocluster might be smaller we assumed. At the same time, FRET originating from proteins that are not in nanoclusters might be smaller than the assumed 10% efficiency. Altogether, the estimates of proteins in microdomains/nanoclusters might be lower estimates.

In further support of our approach, we analysed our data on the dependence of the FRET efficiency on the donor mole fraction (at constant cA) with a model for FRET in oligomers (D.A. and H.V., unpublished data). Nanoclusters have a weak resemblance to oligomers, in that they contain a characteristic number of proteins (Plowman et al., 2005; Sharma et al., 2004). Intriguingly, we determined three to six proteins in a hypothetical oligomer or nanocluster, which was again in full agreement with the two to seven proteins previously determined for nanoclusters (Plowman et al., 2005; Sharma et al., 2004). Also, our estimate of the absolute number of microdomains (3400-16,200) in a cell is comparable with data from active K-ras where ~46,000 microdomains were calculated (Tian et al., 2007).

Our FRET data correlate very well with the EM-clustering analysis of H- and K-ras membrane anchors (Plowman et al., 2005; Prior et al., 2003), which supports our view that the $E_{\text{max}}$ value is a good measure for nanoclustering. The relative differences of the $E_{\text{max}}$ values allowed conclusions about the co-clustering of our FRET pairs of anchor constructs. At some variance with the EM analysis (Prior et al., 2003), the $E_{\text{max}}$ value of the tH/tK pair suggests some co-clustering in HEK293 cells. FRET analysis may therefore appear more sensitive than EM analysis. However, this comparison cannot easily be made, because the FRET and EM analyses are sensitive for clustering on different, partially overlapping (Hess et al., 2005), length scales, namely 3-10 nm and above 5 nm, for FRET and EM respectively. However, it is possible that low levels of co-clustering cannot be detected using the EM analysis. Alternatively, the protein distribution of tH/tK in HEK293 may not exactly match that of BHK cells. Either way, our dynamic range of $E_{\text{max}}$ values remains valid, because other FRET pairs, such as mCFP-tR/N$_2$Cy-mCit (Fig. 3C) or mCFP-rR/N$_2$Cy-mCit (Fig. 4C) show $E_{\text{max}}$ values close to the predicted value for randomly distributed donors and acceptors.

Our data of membrane anchors indicate that heterotrimers of different G-protein subclasses reside in microdomains (or nanoclusters) that

![Fig. 6. FRET experiments with full-length heterotrimeric-G-protein constructs confirm displacement of the Go subunit into another microdomain after activation, as predicted by the FRET vectors of the anchor constructs. (A) Schematic representation of fluorescent full-length heterotrimeric-G-protein constructs. We fused the fluorescent protein (FP) to the N-terminus of the Go subunits and targeted these fusion constructs using the targeting sequences of the G-protein-anchor constructs. (B) Confocal imaging confirmed that all full-length Go constructs are predominantly localized to the plasma membrane. Bars, 10 µm. (C) FRET changes were calculated after stimulating cells with AlF$_4^-$ (30 µM, 40 minutes, 22°C). Fluorescence of cells co-expressing indicated full-length G-protein constructs, microdomain markers and in addition unlabelled GB1γ2, was measured in a sensitive spectrofluorometer. Numbers at the bars give the average FRET change in percent with standard deviations and number of independent experiments, n, in brackets.](image-url)
considerably overlap (Fig. 5B). This would allow for the promiscuity of receptor-heterotrimer interactions often observed. However, the membrane-anchor-specific separation of activated Gα-proteins (Fig. 5B), could provide an explanation for a membrane-anchor-specific and subclass-specific (subclassification is to a large extent determined by the N-terminal membrane anchor) use of effector. The membrane-anchor-specific lateral segregation of signaling proteins would support similar observations made for Ras (Prior et al., 2001). Ras data furthermore suggest the existence of at least three types of microdomain (Prior et al., 2003; Roy et al., 2003). This again parallels our results, as we postulate at least three distinct microdomains for Gαi3, Gαq and the heterotrimers that are, however, again different from the microdomains labelled by our microdomain markers (Fig. 5B).

The usefulness of membrane-anchor data is validated by a study using single-particle tracking, which revealed very similar diffusion modes for full-length heterotrimeric Gα and Gαβγ constructs, and corresponding membrane anchor constructs (Perez et al., 2006). It furthermore provides an interesting correlation of diffusion data on membrane sheets and FRET data in living cells.

The strongest support for our approach comes from our FRET data on stimulated full-length heterotrimeric G proteins (Fig. 6C). The FRET map summary, derived from membrane anchor data (Fig. 5B), allowed to predict and interpret the results in Fig. 6C, which suggest that heterotrimeric G1 and G2 proteins dissociate to an extent that allows the membrane anchor on Gα to induce a different microdomain for its subunit. This is in line with the standard model of G-protein action, which is supported by several other FRET studies (Azpiazu and Gautam, 2004; Gibson and Gilman, 2006; Janetopoulos et al., 2001), but difficult to reconcile with reports proposing that only Gα and not Gβγ proteins dissociate (Frank et al., 2005).

In search of molecular determinants for the observed microlocalization, we propose that a polybasic sequence in conjunction with an isoprenylation leads to co-clustering. Our microlocalization, we propose that a polybasic sequence in conjunction with an isoprenylation leads to co-clustering. Our data furthermore suggest the existence of at least three microdomains, thus providing a more powerful alternative to cholesterol-depletion experiments, which are also error prone due to unspecific effects of cholesterol-depleting agents (Shvartsman et al., 2006). Our data support distinct complexes formed by lipid anchors, surrounding amino acids and membrane lipids, which might induce dynamic, partially overlapping nanoscale assemblies, and might also depend on cholesterol. We believe that this revised model is more suitable to explain the lateral segregation of the plethora of membrane-anchoring motifs, which mostly serve specific cell biological purposes (Fivaz and Meyer, 2003) and might quite possibly not only be confined to two types of microdomain. Our relational FRET map approach will allow to address unresolved questions of membrane microdomains of membrane-anchored or membrane-spanning proteins, such as coupling of domains between two leaflets and modulating effects of putatively membrane-interacting amino acids.

Materials and Methods
Molecular cloning
To construct the microdomain markers, the minimal membrane-anchoring sequences of murine H-ras, K-ras 28, Rac1 or of the G protein Gq2 subunit were C-terminally fused to mCFP and mCt [A206K mutation (Zhang et al., 2002) of cCFP (Clontech) and the improved YFP citrus (Griesbeck et al., 2001, respectively). The G-protein-anchor constructs were generated by fusing the anchoring sequences of the murine G protein subunits Gαi2 and Gαq, murine Lyn and human GAP-43 to the N-terminus of fluorescent proteins, which for Gαiγ-anchor constructs contained also the Gγ2 anchor. Full-length heterotrimeric G protein constructs were constructed as described by others (Leaney et al., 2002), but by using the indicated membrane-targeting sequences (see supplementary material Table S1).

Cell culture
Adherent human embryonic kidney (HEK293) cells were grown in Dulbecco’s modified Eagle’s medium F-12 (Invitrogen), supplemented with 2.2% fetal calf serum. Cells were cultured in plastic flasks (TPP AG, Trasadingen) at 37°C and 5% CO2. Transfection was done using Effectene transfection reagent (Qiagen).

Fluorescence measurements by flow cytometry
Cells were harvested 2 days after transfection with EDTA (5 mM, pH 7.4) washed with PBS (Sigma) and resuspended in PBS. We used a CyAn ADP flow cytometer (Dako Cytomation) to measure fluorescence of 1×105 to 2×105 events in the donor (405 nm excitation, 450/50 emission filter), acceptor (488 nm excitation, 530/40 emission filter) and FRET (405 nm excitation, 530/40 emission filter) channels, with detector gains set to accommodate the full range of expression levels. Raw data were imported into IgorPro5 (WaveMetrics) for subsequent data treatment. We calculated the normalized acceptor surface concentration using the equation cA=NA/AcellR02. Therein, NA is the number of acceptor fluorophores derived from normalizing fluorescence in the acceptor channel with the fluorescence signal of the cellular background. This was conservatively estimated to correspond to 10,000 molecules. This was verified with fluorescent giant unilamellar vesicles of known concentration of fluorophore on their surface (data not shown). Acell is the surface area of a HEK cell estimated as a sphere with the average diameter of 15.4±2.4 μm (n=70). A fusion construct of mCFP-mCt was used to calibrate for a 1:1 stoichiometry of donor:acceptor (corresponding to xD=0.5±0.17). We have selected in silico for those cells, with the donor mole fraction of xD=0.5±0.17 (corresponding to donor:acceptor=1:1 stoichiometry) and used only this population for subsequent data analysis. FRET was calculated per cell by using an adapted sensitized acceptor-emission method (Gordon et al., 1998), using E(mCFP-mCt)=0.35±0.03 for calibration of the conversion factor G. Dependence of the FRET efficiency E on the normalized acceptor surface concentration cA was fitted, using the following:

\[ E = \frac{cA}{1 + cA/G} \]

where G is the calibration of the conversion factor G. Dependence of the FRET efficiency E on the normalized acceptor surface concentration cA was fitted, using the following:

\[ E = \frac{cA}{1 + cA/G} \]
descriptive function, which is adapted from the one used by Wolber and Hudson (Wolber and Hudson, 1979):

$$E = E_{\text{max}} - [E]_{0} e^{-k_{1} \cdot c_{A}^*} + [E]_{0} e^{-k_{2} \cdot c_{A}^*}$$

with $[E]_{0} = 0.6322$, $k_{1} = 3.8171$, $k_{2} = 0.3678$, $c_{A} = 0.7515$, for $R_{D}/R_{A} = 0.7$; using $R_{A}$=4.7 nm, the Förster radius of mCFP/mCit, calculated using spectroscopic data of the purified proteins (D.A., unpublished data) and $R_{c}=3.4$ nm. $R_{c}$ was the distance of closest approach of the fluorescent proteins, which was estimated by modeling them as cylinders, of 2.5×4 nm, with the transition dipole moment in its center (Griesbeck et al., 2001; Rosell and Boixer, 2003). Because the relative orientation of the membrane-anchored fluorophores is not known, $R_{c}$ was calculated as an arbitrarily chosen weighted average of the distances of the dipole moment of three different orientations of the fluorophores. Orientation 1 was side-by-side; $R_{c}(i)=2.5$ nm, orientation 2 was top-on-bottom, $R_{c}(ii)=4$ nm, $x(i)=0.25$; orientation 3 was edge-on-edge, $R_{c}(iii)=4.6$ nm, $x(iii)=0.25$. $R_{c} = x(i)R_{c}(i) + x(ii)R_{c}(ii) + x(iii)R_{c}(iii) = 3.4$ nm. The free fit parameter $\alpha$, the offset on the ca axis $c_{A}^*$ and the maximum efficiency reached at high $c_{A}^*$ $E_{\text{max}}$, resulted from our adaptation.

We calculated the fraction of molecules present in microdomains with the most dense cubic packing was calculated using equation 2:

$$E_{\text{max}} = x_{\text{micro}}E_{\text{micro}} + (1-x_{\text{micro}}) E_{\text{bulk}}$$

with $x_{\text{micro}}=85\%$ being the efficiency in the microdomains at the most dense packing, calculated with $E=1/(1+(R/R_{0})^{6})$, using $R_{0}=4.7$ nm $R=R_{c}=3.4$ nm (see supplementary material Fig. S1B for a table of $x_{\text{micro}}$).

Confocal microscopy and sensitized acceptor FRET imaging HEK293 were plated on coverglasses (0.18 mm), overlaid with PBS and imaged using a LSM 510 confocal microscope (Zeiss) equipped with appropriate filtersets for CFP and YFP imaging. FRET was measured as sensitized acceptor emission using the three cube method essentially as described by others (Gordon et al., 1998). Images in donor (excitation 405 nm, emission 470-500 nm), acceptor (excitation 514 nm, emission 530-600 nm) and FRET (excitation 405 nm, emission 530-600 nm) channels were acquired on a Zeiss LSM 510 Meta using a 63× or 100× oil immersion objective, with numerical apertures of 1.4. The acceptor channel was adjusted to a setting corresponding to -500-2000 acceptors/μm², using giant unilamellar vesicles with 1000, 2000 and 4000 FITC/μm², and taking into account that the brightness of FITC is ~3.8 times smaller than of mCitrine at 514 nm excitation (based on their extinction coefficient and quantum yields). Similar to the cytoplast approach, the mCFP-mCit fusion protein was used to calibrate for the FRET efficiency and donor-acceptor stoichiometry. Images were analysed using a custom written procedure in ImagePro5, that performed background subtraction, crosstalk correction, shifting of correlated three-cube-images and, if required, thresholding. FRET was calculated on a pixel-by-pixel basis. Per image, three regions of interest on the plasma membrane were chosen.

Fluorescence measurements on a spectrofluorimeter Fluorescence measurements were done on a SpexFluorolog II (Instrument S.A.) at 1.60 nm excitation-band- and 0.90 nm emission-band passes. Stirred cell suspension was in a quartz cuvette (10×4 mm², 1500 μl). HeLa cells were excited consecutively at 430 nm and 510 nm, with emission being recorded from 440-600 nm and 520-600 nm, respectively. FRET was determined as ‘FR’, the sensitized acceptor emission FRET ratio; $FR_{R_{D}R_{A}F_{A}}$, where $F_{A}$ and $F_{R}$ are the fluorescence of the acceptor in the presence and absence of the donor, respectively. We derived FR from $FR_{R_{D}R_{A}F_{A}}$, given that the recorded sample spectra are: $S_{x}(x) = a_{x0} e^{-k_{2} \cdot c_{A}^*}$ being the fluorescence signal of y (e.g. acceptor or donor) of the sample containing x (e.g. acceptor and donor) after excitation at λex nm; f1-f2 were the spectra of reference samples as indicated in brackets, recorded by excitation at the indicated wavelengths; coefficients $a_{x0}$ to $d_{x0}$ are the fit parameters. The percent FRET change after AlF₄⁻ treatment (30 μM, 40 min, 22°C) was calculated as: $(FR_{x_{0}}-FR_{x_{0}})/FR_{x_{0}}$. We thank Andrew Tinker for the kind gift of the parent constructs of the membrane targeted, fluorescent Go₁₁₆₆ proteins, Peter Ulrich for help with programming, Bruno Meyer and Jean-Manuel Segura for valuable discussions, and Ruud Hovius for critical reading of the manuscript. D.A. is grateful to John Hancock for his helpful advice on the manuscript. This work was supported by the Swiss National Science Foundation (NF 4047-057572), the TopNano21 program (projects 5636.2 and 6266.1), and internal grants from the EPFL.

References


**Table S1. List of primers for PCR-addition or amplification of indicated membrane targeting sequences.**

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List of primers for PCR-addition or amplification of indicated membrane targeting sequences. N- or C-terminal membrane targeting sequences were added either by two sequential PCRs or by molecular cloning of PCR-amplified cDNA fragments encoding the targeting sequence. Final constructs were in pcDNA3, pcDNA3.1 (Invitrogen) or in the backbones of pEYFP-C1 or pECFP-N1 (BD Biosciences/Clontech). We mutated eYFP (BD Biosciences/Clontech) to Citrine (=eYFP Q69M) using: 5'-CGTGACCACCTCGCTACGGATTATGCTCTCCCGCTACCC-3' (45 bp, sense) and the complementary antisense primer. The cDNAs of eCFP (BD Biosciences/Clontech) and Citrine were mutated site directedly to mCFP and mCit, respectively, by introducing mutation A206K (Zacharias et al., 2002; Zhang et al., 2002) using mutagenising primers with the following sequences: 5'-CCTGAGCACCCAGTCCAAGCTTAGCAAAGACCCCAACG-3' (sense for eCFP as a template) and 5'-CCTGAGCTACCAGTCCAAGCTTAGCAAAGACCCCAACG-3' (36 bp, sense for Citrine as a template). All oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

*the first of the six lysine residues (bold) was the terminating amino acid of the fluorescent protein.

ms, mouse; hu, human.
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