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# Discrepancy between fluorescence correlation spectroscopy and fluorescence recovery after photobleaching diffusion measurements of G-protein-coupled receptors

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ABSTRACT

Fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS) are the two most direct methods to measure the diffusion of molecules in intact living cells. Ideally, these methods should produce similar results for an identical system. We have used these methods to monitor the diffusion of two G-protein-coupled receptors and their associated proteins in the plasma membranes of cells that do not or do contain invaginated protein domains called caveolae. FRAP studies show that caveolae domains increase the immobile fraction of receptors without significantly changing their mobility. On the other hand, FCS studies show an unexpected increase the mobility of caveolae-associated proteins. Our data suggest that the geometry of caveolae domains gives rise to a confined diffusion of its attached proteins, resulting in an apparent increase in mobility.



Fluorescence correlation spectroscopy (FCS)[1] and fluorescence recovery after photobleaching (FRAP) are routinely used to measure the diffusion of fluorescent proteins in cells. FRAP monitors the recovery of fluorescence by the diffusion of fluorophores into a region that has been bleached by a high-intensity laser. Usually, the bleach spot is on the micron scale and the recovery is more than 1 min, depending on the mobility of the fluorophore [1]. FCS, on the other hand, monitors the fluctuations of fluorescence intensity as molecules diffuse in and out of a small (~1 fl) confocal volume [2]. The most common type of FCS measurement is single-point FCS, which has the drawback of being sensitive only to diffusing fluorophores while immobile ones are not detected. Alternately, FRAP measurements give a good indication of the population of species that are immobile during the sampling period. In principle, FCS and FRAP should offer similar and complementary information. However, because the size of the sampling areas differs greatly in the two methods, discrepancies may arise due to local structural barriers that impede or corral the movement of probes. This is particularly true on the plasma membranes of living cells where diffusion barriers exist (see, e.g., Ref. [3]).

Here, we have used FCS and FRAP to determine the effect of membrane domains called caveolae on the diffusion of two related integral membrane proteins. Caveolae are flask-shaped membrane invaginations (see Fig. 1 and Refs. [4–6]) formed from the caveolin family of proteins (see Refs. [6–11]). Caveolae are found on the plasma membrane of many mammalian cells. These domains appear to participate in vesicle trafficking and endocytosis. In addition, caveolin proteins (i.e., Cav1 and Cav3) may specifically bind to other cellular proteins involved in transmission of extracellular signals (see, e.g., Refs. [12,13]).

An important class of signaling proteins that may target caveolae are G-protein-coupled receptors (GPCRs) [14]. GPCRs are the largest family of mammalian receptors that structurally consist of seven transmembrane helices. When an extracellular agent interacts with its specific GPCR, it initiates a series of sequential molecular interactions that involve activation of surface-associated heterotrimeric G proteins and subsequent activation or inhibition of cytosolic enzymes that result in various cellular responses [15]. Many GPCRs and G-protein subunits have been reported to localize in lipid rafts and caveolae domains (see Refs. [14,16]). By corralling GPCRs and G proteins, caveolae may affect signaling by promoting their oligomerization, their association with agonists, and their interaction with intracellular G proteins.

Heterotrimeric G proteins are activated by GPCRs and consist of a Gα and a Gβγ subunit. There are four families of Gα subunits, and

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[1] *Abbreviations used:* FCS, fluorescence correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; GPCR, G-protein-coupled receptor; FRT, Fisher rat thyroid; $B_2R$, bradykinin type 2 receptor; PLCβ, phospholipase Cβ; FRET, Förster resonance energy transfer; eCFP, enhanced cyan fluorescent protein; eYFP, enhanced yellow fluorescent protein; μOR, μ-opioid receptor; eGFP, enhanced green fluorescent protein; vrFRAP, variable radius FRAP; N&B, number and brightness.

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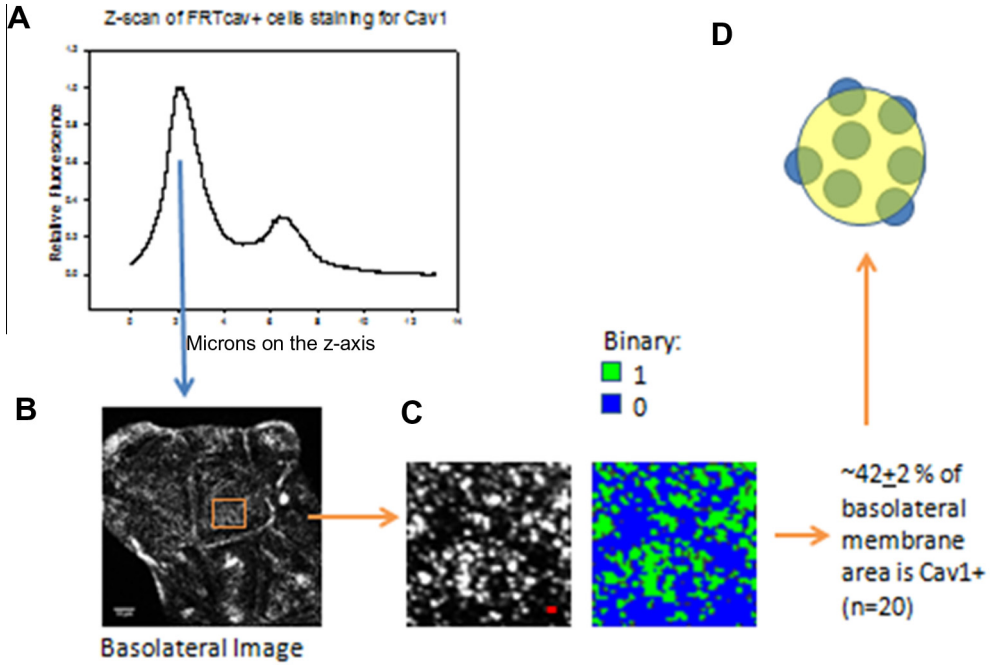


**Fig.1.** (A) Example of the distribution of Cav1–eGFP in the *Z* direction expressed in an FRTwt cell. (B) Corresponding image of the cell. (C) Expanded view of a region of the image in black and white and binary depiction. (D) Cartoon depicting caveolae in an FCS-based illuminated measurement.

only the $G\alpha_q$ subtype has been reported to reside in caveolae domains [16]. Our laboratory used live cell fluorescence imaging to show that in the basal state, $G\alpha_qG\beta\gamma$ localizes to caveolae domains [17] due to strong interactions between $G\alpha_q$ and Cav1 [17,18]. For those studies, we used Fisher rat thyroid (FRTwt) cells with corroborating experiments in other cell lines. FRTwt cells do not express detectable levels of Cav1, but a sister line that is stably transfected with canine Cav1 (FRTcav+) displays caveolae domains as visualized by electron microscopy [7,19]. In this cell line, caveolae appear at high density on the basolateral membrane and very little on the apical membrane. Furthermore, caveolae are enriched in regions of cell-to-cell contact in accord with the observations that they may organize proteins involved in intercellular signaling such as connexins [20].

$G\alpha_q$ is coupled to many GPCRs, and its activation results in an increase in intracellular calcium, resulting in mitogenic and proliferative changes in the cell (see Ref. [15]). One of the more notable GPCRs that is coupled to $G\alpha_q$ is the bradykinin type 2 receptor ($B_2R$). $B_2R$ binds the extracellular agonist bradykinin, which is a key mediator of the inflammation response [21]. The binding of bradykinin to $B_2R$ activates $G\alpha_q$, resulting in activation of phospholipase Cβ (PLCβ) enzymes that ultimately results in an increase in intracellular calcium and activation of many calcium-sensitive proteins. FRT cells do not express $B_2R$ receptors, allowing us to monitor the effect of caveolae domains on the homo-oligomerization of these receptors.

We have found that the presence of caveolae greatly affects $B_2R$/$G\alpha_q$ signaling, correlating with a significant increase in calcium release in FRTcav+ cells as compared to FRTwt [17,20]. In addition, we have found a significant amount of Förster resonance energy transfer (FRET) between eCFP–Cav1 and $B_2R$–eYFP and between enhanced cyan fluorescent protein (eCFP)–Cav1 and $G\alpha_q$–eYFP (enhanced yellow fluorescent protein), supporting a caveolae localization of these proteins [17,20]. These studies, as well as sedimentation studies, suggest that $B_2R$ localizes to caveolae domains [22]. In contrast, the presence of caveolae does not affect the function of another GPCR pathway, the μ-opioid receptor (μOR)/$G\alpha_i$ system, correlating with a lack of FRET between these proteins and Cav1 [20].

Here, we have measured the impact of caveolae on the diffusion properties of $B_2R$ using FCS and FRAP. Although FRAP studies show a small increase in the immobile population of $B_2R$ in the presence of caveolae, FCS studies show an unexpected increase in receptor mobility with caveolae. We postulate that this surprising FCS result is caused by confined movement of $B_2R$ to the periphery of caveolae domains.

## Materials and methods

### *Materials*

FRTwt and FRTcav+ cells and canine Cav1–eGFP (enhanced green fluorescent protein) DNA were gifts from Deborah Brown (Stony Brook University) and were cultured in F-12 Coon's modified medium obtained from Sigma, as described previously [17]. Cells were imaged in Leibovitz's L-15 medium from Gibco (see Ref. [23]) and transfected using Lipofectamine (Invitrogen) following the manufacturer's protocol.

μOR–eGFP and $G\alpha_i$–eYFP were obtained from Lakshmi Devi (Mount Sinai Medical Center). $G\alpha_q$–eYFP was obtained from Catherine Berlot (Geisinger Research). $B_2R$ and $B_2R$–GFP were obtained from Fredrik Leeb-Lundberg (Lund University). We have found that expressed proteins are functional [17,20,24]. The membrane marker is an eYFP fused with the first 20 amino acids of Gap-43 and is palmitoylated on cysteines 3 and 4 posttranslationally (Clontech).

### *FRAP measurements*

FRTwt and FRTcav cells expressing fluorescently tagged proteins were seeded on glass-bottom dishes (MatTek). Cells were imaged with a 60× oil objective (NA 1.42) using an Olympus FluoView FV1000 microscope. For variable radius FRAP (vrFRAP), the focal plane was set on either the top or bottom membranes