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Z-scan Fluorescence Profile Deconvolution of Cytosolic and Membrane-associated Protein Populations

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Abstract

This study introduces a technique that characterizes the spatial distribution of peripheral membrane proteins that associate reversibly with the plasma membrane. An axial scan through the cell generates a z-scan intensity profile of a fluorescently labeled peripheral membrane protein. This profile is analytically separated into membrane and cytoplasmic components by accounting for both the cell geometry and the point spread function. We experimentally validated the technique and characterized both the resolvability and stability of z-scan measurements. Further, using the cellular brightness of green fluorescent protein, we were able to convert the fluorescence intensities into concentrations at the membrane and in the cytoplasm. We applied the technique to study the translocation of the pleckstrin homology domain of phospholipase C-delta1 labeled with green fluorescent protein upon ionomycin treatment. Analysis of the z-scan fluorescence profiles revealed protein-specific cell height changes and allowed for comparison between the observed fluorescence changes and predictions based on the cellular surface area to volume ratio. The quantitative capability of z-scan fluorescence profile deconvolution offers opportunities for investigating peripheral membrane proteins in the living cell that were previously not accessible.

Keywords

peripheral membrane protein; point spread function; brightness; translocation; phospholipase C; Pleckstrin homology domain

INTRODUCTION

Peripheral membrane proteins associate reversibly with cellular membranes through noncovalent interactions with lipids and integral membrane proteins. They constitute a broad

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and diverse class of proteins involved in various membrane-mediated cellular processes including but not limited to cell signaling, cytoskeletal structure, lipid homeostasis, and electron transport [1–3]. The ability to transition between a soluble and membrane-bound form has been recognized as an important regulation and control mechanism of cells. The interaction with the membrane provides a mechanism to induce conformational changes in the protein that modulate its activity. Vice versa, the interactions with the membrane-associated protein can also change the composition, dynamics, and shape of cellular membranes [4–7]. This complex, bidirectional interplay between peripheral membrane proteins and cellular membranes is only beginning to be unraveled through the use of model membrane systems and cellular studies.

The energetics and dynamics of the interaction process has been primarily studied with model membranes [8,9]. These experiments provide quantitative information about protein interactions with lipids, but like all *in vitro* studies cannot reproduce the environment and complex interaction network found in cells. Live-cell studies monitor peripheral membrane proteins in their natural habitat and are an essential complement to *ex situ* methods. Cellular translocation studies of fluorescent protein-tagged proteins provide a powerful and convenient approach to visualize the subcellular distribution of peripheral membrane proteins and their dynamic relocation in real time by fluorescence imaging methods, such as confocal or TIRF microscopy [10–13].

Translocation studies typically report qualitative differences in the subcellular distribution of proteins, which reflects the difficulty of identifying the concentration of the membrane-bound and soluble protein from fluorescence intensity data [10,11]. Attempts at quantifying the fluorescence signal to gain information about the relative strength of the interaction between protein and membrane have been restricted to heuristic approaches that are unable to analytically separate the fluorescence signal. The current study addresses this problem and introduces a fluorescence-based approach for measuring the time-resolved distribution of proteins at the plasma membrane and in the cytosol of a living cell.

We specifically focus on characterizing the interaction of a fluorescently labeled cytoplasmic protein with the plasma membrane. An axial scan of the two-photon point spread function (PSF) through the cell generates a z-scan intensity profile as illustrated in Fig. 1. By accounting for the cell geometry and the PSF we recover the distinct cytoplasmic and membrane-bound protein fluorescence contributions from the intensity profile, a process we refer to as z-scan fluorescence profile deconvolution (FPD). The intensity profile was first introduced in z-scan fluorescence fluctuation spectroscopy (FFS) studies [14,15] where it served as a byproduct for determining the proper weighing factors that connected the FFS data to the oligomeric state of proteins at the plasma membrane and in the cytoplasm. Here we focus exclusively on the z-scan intensity profile to demonstrate that it offers a powerful approach for studying protein binding at the plasma membrane without the need for time-consuming FFS measurements.

We first examined the resolvability limits of FPD analysis of the fluorescence intensity profile by systematically varying the cytoplasmic intensity relative to the membrane fluorescence intensity and studied the stability of z-scan measurements over a prolonged

sampling time. We applied the technique to investigate the translocation of the green fluorescent protein labeled pleckstrin homology (PH) domain of Phospholipase C-delta1 (PH-PLC δ -EGFP) in U2OS cells from the plasma membrane to the cytoplasm upon treatment with ionomycin. Quantitative analysis of the z-scan intensity profiles taken before and after treatment identified movement of the membrane position and allowed direct comparison between the fluorescence change at the membrane and in the cytoplasm.

MATERIALS AND METHODS

Experimental Setup

All data were taken with a Zeiss 63x C-Apochromat water immersion objective (N.A.=1.2) on a home-built two-photon microscope as described previously [14,16]. The sample was excited at a wavelength of 1000 nm and an average power ranging from 0.30–0.38 mW after the objective. The dual-channel measurements were carried out with a dichroic mirror centered at 580 nm to split the fluorescence emission into two detection channels. An additional 84-nm-wide bandpass filter centered at 510 nm was added to the green detection channel (Semrock, Rochester, NY) to eliminate the reflected fluorescence of mCherry. Photon counts were detected by an avalanche photodiode (APD, SPCM-AQ-141, Perkin-Elmer, Dumberry, Quebec) and recorded by a Flex02-01D card (correlator.com, Bridgewater, NJ). Data analysis was performed in IDL 8.3 (Research Systems, Boulder, CO).

An arbitrary waveform generator (Model No. 33250A, Agilent Technologies, Santa Clara, CA) provided the voltage signal to drive a PZ2000 piezo stage (ASI, Eugene, OR) along its z-axis. A triangular function with a peak-to-peak amplitude of 2.4 V and a period of 10 seconds was used for the z-scan experiments. This waveform corresponded to 24.1 μ m of axial travel in 5 s with the cells occupying roughly 3–4 μ m in the center of each pass. The data sampling frequency of the z-scan experiments was 20 kHz. In addition, we performed independent measurements on cells expressing the fluorescent label EGFP to determine its brightness λ_{EGFP} as previously described [17,18]. This brightness serves as a calibration factor for the conversion of fluorescence intensities into concentrations.

Plasmid Construction, Sample Preparation, and Cell Measurements

The pEGFP-C1 and pEGFP-N1 plasmids were purchased from Clontech (Mountainview, CA), and the mCherry-C1 plasmid has been previously described [19]. The EGFP-H-Ras plasmid was a gift from Dr. Mark Phillips (New York University School of Medicine). The PH-PLC δ -EGFP plasmid was a gift from Dr. Joseph Abanesi (University of Texas-Southwestern). All sequences were verified by automatic sequencing. The cellular studies were performed using transiently transfected U2OS cells (HTB-96) that were obtained from ATCC (Manassas, VA) and maintained in 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) and DMEM medium. Cells were subcultured in eight-well coverglass chamber slides (Nalge Nunc International, Rochester, NY) 12 hours before transfection. Transient transfections were carried out using GeneJet (Thermo Scientific, Pittsburgh, PA) according to the manufacturer's instructions, 24 hours prior to measurement. Immediately before

measurement, the growth medium was replaced with 200 μL of Leibovitz's L-15 Medium, no phenol red (Gibco, Life Technologies, Grand Island, NY).

For kinetic studies, repeated z-scans were performed on individual cells. An objective heater with a thermal controller (MATS-ULH, Tokai Hit, Japan) was used to maintain a medium temperature of 32°C. Prior to scanning, 100 μL of medium was removed from the well. Pre-warmed L-15 medium (100 μL) containing Texas Red and ionomycin calcium salt (Life Technologies, Carlsbad, CA) was later added to the well, for a final concentration of 1.26 mM for Ca^{2+} and 5 or 10 μM for ionomycin. Texas Red served as a tracer to identify the time of delivery of ionomycin at the cell and was monitored in the red detection channel.

The delta layer model was created by using a layer of rhodamine fibronectin (Cytoskeleton, Inc, Denver, CO). The fibronectin layer was created by pipetting a solution of 200 μL of buffer (0.5M NaCl, 0.5M Tris, pH=7.5) mixed with 13 μg of fibronectin into a chamber of an eight-well coverglass slide. The 8-well was then placed in an incubator for 24 hours before the remaining liquid was pipetted out and replaced with 200 μL of Dulbecco's phosphate-buffered saline (PBS) with calcium and magnesium (Biowhitaker, Walkerville, MD). For the semi-infinite model, a dilute fluorescein solution was added to a well of the chamber slide.

Z-scan Calibration of PSF

A modified squared Gaussian-Lorentzian (mGL) point spread function,

$$PSF(\rho, \zeta) = \left(\frac{z_0^2}{z_0^2 + \zeta^2} \right)^{(1+y)} \exp \left(-\frac{4z_0^2}{w_0^2} \frac{\rho^2}{z_0^2 + \zeta^2} \right), \quad (1)$$

first introduced by Macdonald et al. [14], provides a good approximation of the PSF of our two-photon microscope and serves as the model for this study. A z-scan calibration procedure was performed as described previously [14] to determine the free parameters of our model. The calibration resulted in $z_0 = 1.02 \pm 0.1 \mu\text{m}$, $y = 2.50 \pm 0.3 \mu\text{m}$, and $w_0 = 0.47 \pm 0.05 \mu\text{m}$, where w_0 and z_0 describe the radial and axial beam waist, while y changes the axial decay shape of the PSF. Z-scan analysis relies on the radially integrated point spread function RIPSF, which for the mGL PSF is given by [14],

$$\text{RIPSF}(z) = \int_0^\infty PSF(\rho, \zeta) 2\pi\rho d\rho = \frac{\pi w_0^2}{4} \left(1 + (z/z_R)^2 \right)^{-y}. \quad (2)$$

The mGL PSF volume is given by $V_\infty = \int_{-\infty}^{+\infty} \text{RIPSF}(\zeta) d\zeta = 1/4(\pi w_0^2 z_R) \sqrt{\pi} \Gamma(y - \frac{1}{2}) / \Gamma(y)$,

and the cross-sectional area at the center of the mGL PSF is determined by $A_0 = \frac{\pi w_0^2}{4}$ [14].

z-scan Data Analysis

Photon counts were rebinned by software to a z-scan sampling time of $T_z = 4 \text{ ms}$, which with a z-scan speed of $v_z = 4.82 \mu\text{m/s}$ provides a step size $z = v_z T_z = 19.3 \text{ nm}$ between

adjacent binned photon counts k_z . The experimental fluorescence intensity profile is given by $F(z) = k_z/T_z$ and fit to a model intensity profile using a Levenberg–Marquardt algorithm with the PSF parameters z_R and y fixed to the calibrated values. The standard error σ of k_z was determined from the standard deviation of the unbinned counts by $\sigma = \sigma_{unbinned} \sqrt{N_B}$ with the number of samples N_B in a single bin being equal to 80.

Simulations were performed as follows. A z-scan intensity profile $F_{\text{model}}(z)$ for a membrane-cytoplasmic-membrane geometry was calculated from Eq. 6 using the calibrated PSF parameters and experimental step size Δz . The calculated intensity profile was converted into simulated data $F_{\text{sim}}(z)$ using a Poissonian random number generator $P_{\text{Poisson}}(\lambda)$ to

$$\text{account for shot noise, } F_{\text{sim}}(z) = \frac{P_{\text{Poisson}}(F_{\text{model}}(z) \cdot T_z)}{T_z}.$$

RESULTS

The z-scan intensity profile of single layers

A scan of the two-photon excitation spot along the z-axis of a cell generates a fluorescence intensity profile $F(z)$. This intensity profile results from the convolution of the PSF of the instrument with the concentration profile $c_M(z)$ of the fluorescently labeled protein along the scan axis. We assume in this study that the concentration only varies along the z-direction, which reflects a geometry based on stratified layers. For a single layer a scan along the z-direction results in a fluorescence intensity function [14]

$$F(z) = \lambda c \int_{-\infty}^{\infty} S(\zeta + z) \text{RIPSF}(\zeta) d\zeta, \quad (3)$$

where λ is the monomeric brightness of the labeled protein, c represents the concentration of the labeled protein expressed as monomers, and RIPSF is the radially integrated point spread function [14]. The function $S(z)$ describes the geometric shape of the single layer [14] and will be discussed in more detail later. We define the concentration profile as the product of the protein concentration and the geometric shape function, $c_M(z) = cS(z)$.

We consider three basic geometries and their associated intensity profiles: the delta-layer, the slab, and the semi-infinite layer. The concentration profile of the slab layer $c_M(z) = c\Pi(z; z_B, z_T)$ is constant between z_B and z_T (Fig. 2A), which corresponds to a geometric shape function $\Pi(z; z_B, z_T)$ that equals 1 for $z_B \leq z \leq z_T$ and 0 everywhere else. The fluorescence intensity profile for the slab layer is obtained from Eq. 3,

$$F(z) = F_{\infty} \alpha_V(z; z_B, z_T), \quad (4)$$

which introduces the fractional PSF volume $\alpha_V(z; a, b) = \int_{a-z}^{b-z} \text{RIPSF}(\zeta) d\zeta / V_{\infty}$ to describe the incomplete overlap of the PSF with the sample [15]. This description differs from conventional FFS, where we assume a PSF that is completely embedded within the sample. In essence, conventional FFS corresponds to a shape function $S(z) = 1$ and a constant concentration profile $c_M(z) = c$. In this case Eq. 3 reduces to a z-independent fluorescence

intensity, $F_{\infty} = \lambda c V_{\infty}$. Conversely for a thinner cell section, the PSF only achieves partial overlap with the sample, which is accounted for by a fractional PSF volume $V(z) = V_{\infty} \alpha_V(z)$. The z-scan intensity profile through a cell expressing a fluorescently labeled cytoplasmic protein is well approximated by a fit to the slab model (Fig. 2B). The maximum intensity $F_{\max}$ of the z-scan occurred at the midsection between the bottom and top layer, $z_{mid} = \frac{1}{2}(z_B + z_T)$, and is less than the limiting value of F_{∞} , because the cell thickness at the scan location is not sufficiently thick to completely embed the PSF ($\alpha_V(z_{mid}; z_B, z_T) < 1$).

The concentration profile of the semi-infinite layer (Fig. 2C) is similar to the slab layer, but with $z_T \rightarrow \infty$, which leads to an intensity profile $F(z) = F_{\infty} \alpha_V(z; z_B, \infty)$. A good example of such an intensity profile is given by a z-scan through the microscope coverslip into a fluorescent solution as shown in Fig. 2D together with a fit to the model. The intensity increases as the PSF moves deeper into the solution and reaches a maximum of F_{∞} once it is completely embedded in the solution.

The delta-layer describes a very thin section, such as the plasma membrane, with a thickness which is much less than the axial size of the PSF. Its concentration profile is given by $c_M(z) = \sigma \delta(z - z_M)$, where $\delta(z)$ is the delta-function, σ represents the surface concentration of the layer, and z_M is the axial position of the thin layer (Fig. 2E). The intensity profile for a delta-layer located at z_M is obtained from Eq. 3,

$$F(z) = \lambda \sigma \text{RIPSF}(z_M - z) = F_{\max} \alpha_A(z_M - z). \quad (5)$$

The RIPSF value at a specific location z may be interpreted as an area determined by the cross section of the PSF with the delta layer, $A(z) = \text{RIPSF}(z)$. The maximum of the intensity $F_{\max}$ is reached when the PSF is centered on the membrane ($z = z_M$), which corresponds to $F_{\max} = \lambda \sigma_0 A_0$ with $A_0 = A(0)$. We define a fractional PSF area by $\alpha_A(z_M - z) = A(z_M - z)/A_0$ to describe the intensity profile of Eq. 5 in compact form. A coverslip covered with a thin layer of fluorescently labeled fibronectin served as a model system for a delta-layer. A fluorescence intensity z-scan through the sample and its fit to Eq. 5 is shown in Fig. 2F.

The z-scan intensity profile of multiple layers

A peripheral membrane protein found at the plasma membrane and in the cytoplasm is described by a delta-slab-delta concentration profile $c_{dsd}(z)$ consisting of a delta layer for the bottom membrane located at z_B , followed by a slab layer and another delta layer representing the top plasma membrane located at z_T . This concentration profile (Fig. 3A) can be written as $c_{dsd}(z) = \sigma_B \delta(z - z_B) + c_{Cyto} \Pi(z; z_B, z_T) + \sigma_T \delta(z - z_T)$, where σ_B and σ_T denote the surface concentrations of the fluorescently-labeled protein at the bottom and top membrane, while c_{Cyto} is the cytoplasmic protein concentration. The corresponding fluorescence intensity profile is the sum of the intensity profiles from each layer [15],

$$F_{dsd}(z) = F_B \alpha_A(z - z_B) + F_{Cyto, \infty} \alpha_V(z; z_B, z_T) + F_T \alpha_A(z - z_T). \quad (6)$$

with F_B and F_T being the maximum fluorescence intensity at the membrane layers and $F_{Cyto, \infty}$ represents the limiting cytoplasmic intensity of a thick layer.

We introduce the membrane intensity fraction f_M as a measure for the relative amount of fluorescence intensity coming from the membrane,

$$f_M = \frac{F_M}{F_M + F_{C_{yto},\infty}}, \quad (7)$$

with F_M and $F_{C_{yto},\infty}$ being the maximum fluorescence intensity at the membrane and in a thick cytoplasmic sample as defined by Eq. 6. Because the z-scan intensity profile distinguishes between the bottom and top membrane, we further introduce the intensity fraction f_B of the bottom membrane and the intensity fraction f_T of the top membrane, which are defined by replacing F_M with F_B and F_T in Eq. 7, respectively. High membrane intensity fractions are easy to resolve, because the z-scan intensity profile displays two prominent peaks, as illustrated by the EGFP-H-Ras data (Fig. 3B; $f_B = 0.96$ and $f_T = 0.97$). Conversely, we expect a larger uncertainty in determining f_M from data with lower membrane intensity fractions.

We transfected cells with EGFP-H-Ras and mCherry to experimentally determine the limits of resolvability of f_M . H-Ras is predominantly membrane-bound, while mCherry is entirely cytoplasmic. The presence of two differently colored fluorescent proteins provided a straightforward method to select cells expressing each protein at the desired intensity ratio. This approach allowed us to systematically vary the membrane intensity fraction over a wide range. Because the fluorescence was split into a green and red detection channel, we combined the photon counts of both channels in software to mimic the fluorescence signal of a single-colored fluorescent protein found in the cytoplasm and at the membrane.

We selected two cells that illustrate z-scan intensity traces with low membrane intensity fractions. The intensity profile of Fig. 3C retains a slight double peak, and the fit to the three layer model returns membrane intensity fractions of $f_B = 0.32$ and $f_T = 0.36$. The next intensity profile (Fig. 3D) lacks the double peak, which is consistent with a fit returning lower membrane intensity fractions than in the previous case ($f_B = 0.19$, $f_T = 0.12$). We performed a systematic study by selecting cells based on the relative membrane intensity fraction and performed 10 consecutive z-scans at a thick section of each cell. After fitting the intensity profile of each scan the average and standard deviation of the 10 membrane intensity fractions was calculated and is plotted as stars in Fig. 4A. The experiments were performed on cells with cytoplasmic intensities $F_{C_{yto},\infty}$ ranging from 100 to 1000 kcps and scans were taken at cell heights ranging from 2.2 μm to 4.1 μm (mean of 3.1 μm).

We further performed simulations to compare the experimental uncertainties with predictions based on our model. Intensity traces were calculated according to Eq. 6 with shot noise added to account for the photon detection noise. Parameters were chosen that mimic the experimental conditions as explained in the Materials and Methods section. The cell height was 3 μm for all simulations. The membrane intensity fraction was varied between 0.05 and 0.9 for cytoplasmic intensities of $F_{C_{yto},\infty}$ of 10 kcps, 100 kcps, and 1000 kcps. Multiple traces ($n = 1000$) were simulated for each choice of parameters and analyzed analogous to the experimental data described above to determine the standard deviation of

the membrane intensity fraction. The result of the simulations, plotted as lines with filled symbols in Fig. 4A, demonstrate that higher intensities and higher membrane intensity fraction reduce the uncertainty. Our experimental results closely match the simulation results in that the experimental data are scattered between the simulation results for cytoplasmic intensities of $F_{C_{yto},\infty}$ of 100 kcps and $F_{C_{yto}}$ of 1000 kcps. This suggests that shot noise is the dominant factor shaping the experimental uncertainty. At lower membrane intensity fractions ($f_M \approx 0.1$) the uncertainty increases rapidly, which imposes a practical limit for resolving very small signal contributions from the membrane. Since the experimental and simulated results demonstrate the feasibility of resolving membrane intensity fractions ≈ 0.1 from z-scan intensity profiles, we limited our study to cells with $f_M \approx 0.1$.

Stability of cellular z-scan intensity profiles

The study of time-dependent changes in the membrane-bound population of cellular proteins requires a series of z-scans over a prolonged period of time. Any process that alters the binding conditions of the peripheral membrane protein will be reflected in a time-dependent evolution of the membrane intensity profile. The changes in the cytoplasmic and membrane-bound populations of the protein can be identified from analysis of the z-scan profiles provided that the instrument is stable and introduces no artifacts. We investigate the stability of our setup by performing a series of repeated z-scans through the same x-y position in a cell. By fitting the intensity profiles from the repeated scans to Eq. 6, membrane movement, cell height changes, and instrument z-drift can be studied over time. Fig. 5A shows the top and bottom membrane position of a U2OS cell expressing EGFP-H-Ras over a 750 s period. An offset was added to the data to place the midpoint of the cell initially at zero. The position of the top membrane, drifts by approximately $0.5 \mu\text{m}$ (standard deviation of $0.12 \mu\text{m}$) in parallel with the position of the bottom membrane during the repeated scans. This parallel motion of the two cellular surfaces is indicative of focal drift in the instrument, while the cell height (Fig. 5B), $h = z_T - z_B$, stays approximately fixed (standard deviation of $0.02 \mu\text{m}$). The fastest drift rate estimated from Fig. 5A is 10 nm/s , which is small enough to have a negligible influence on the shape of the intensity profile, because the PSF only has significant overlap with the cell for $\sim 1 \text{ s}$ during the scan.

Despite drift and occasional changes in cell height, the fluorescence intensities determined from fits to Eq. 6 remain remarkably constant as demonstrated by Fig. 5C & D. The average membrane intensity fractions for the top and bottom membrane are $f_T = 0.87$ and $f_B = 0.85$ with a standard error of 1%. The experimental uncertainty agrees well with the simulations from Fig. 4A, which predict an uncertainty of 1% for $F_{C_{yto},\infty} = 10 \text{ kcps}$ for a membrane fraction of 0.9. Because we also collected intensity fluctuations, our experiments were capable of converting fluorescence intensities to concentrations. The relation $F_{C_{yto},\infty} = \lambda c_{C_{yto}} V_\infty$ connects the cytoplasmic concentration and intensity. The PSF volume was calculated as explained in the Material and Methods section and the brightness of the monomeric protein was determined as previously described [15]. Similarly, the surface concentration of the protein at the top and bottom membrane are given by $F_T = \lambda \sigma_T A_0$ and $F_B = \lambda \sigma_B A_0$. Applying these relations we obtained $\sigma_B = 1260 \mu\text{m}^{-2}$, $\sigma_T = 1530 \mu\text{m}^{-2}$ for Fig.

5C and $c_{\text{Cyto}} = 340 \mu\text{m}^{-3}$ for Fig. 5D. The intensity variations over the measurement period of 750 s lead to a relative uncertainty of less than 5% for all concentrations.

The data and simulations illustrate that the uncertainty of determining membrane intensity fraction strongly depend on the fluorescence intensity (Fig. 4A). Thus, quantifying small intensity fractions when the cellular protein concentration is low will be hampered by large uncertainties. Since shot noise appears to be the dominant noise limiting the resolvability of membrane intensity fractions, collecting more photons in the z-scan profile should reduce the uncertainty. Because cell height stays fixed over prolonged periods of time, this suggests the possibility of taking consecutive z-scans and aligning the individual z-scan intensity profiles by software to correct for offsets introduced by focal drift. The photon count signal of the aligned intensity profiles are then added up to create a summed profile with a larger signal, which is expected to result in a reduction in the uncertainty of the fit parameters. To test this procedure and facilitate the early detection of small membrane intensity fractions in cells with low cytoplasmic proteins concentrations, we selected cells with cytoplasmic intensities ranging from 11 to 16 kcps. We collected 100 z-scan intensity profiles from each cell and divided the scans into groups of N , which were aligned and summed. Each of the summed profile was fit to Eq. 6 and the average and standard deviation of the fitted membrane intensity fraction f_M were determined. The inset in Fig. 4A shows the relative uncertainty in f_M of individual scans (open circles, $N = 1$) together with the uncertainty of the summed intensity profiles (filled circles, $N = 10$). Summing 10 intensity profiles lead to an average reduction in the relative uncertainty of 2.8 with a standard deviation of 0.6. The decrease of the relative error with N is illustrated in Fig. 4B for one of the data sets. The results are consistent with the expected $1/\sqrt{N}$ behavior and demonstrate the feasibility to improve the signal-to-noise ratio of dim samples by combining the signal from several fast z-scan intensity profiles.

Kinetic studies of peripheral membrane protein partitioning

Time-dependent changes in the distribution of a membrane-bound protein can be quantitatively characterized through a series of repeated z-scans. Here we study the PH domain of PLC δ which binds with high affinity to PtdIns(4,5) P_2 lipids at the plasma membrane [20]. When fluorescently labeled, PH-PLC δ -EGFP maps the distribution of PtdIns(4,5) P_2 within a cell and exhibits a z-dependent intensity profile with a prominent double peak. Ionomycin induces depletion of internal Ca^{2+} stores and stimulates store-operated Ca^{2+} entry, which raises the Ca^{2+} concentration in the cytoplasm [21]. The increased Ca^{2+} concentration in turn activates PLC-mediated hydrolysis of PtdIns(4,5) P_2 into diacylglycerol (DAG) and inositol triphosphate (IP_3) [22]. This process leads to the displacement of PH-PLC δ -EGFP from the plasma membrane to the cytosol, because the hydrolysis depletes the membrane binding site of the protein [23].

We investigate the time-resolved distribution of PH-PLC δ -EGFP by performing a series of repeated z-scans through the same x-y position in a cell and fitting the intensity profiles from the repeated scans to Eq. 6. Fig. 6A shows the fluorescent intensities from the top membrane, bottom membrane, and cytoplasm over a period of 210 s within the cell. Ionomycin containing solution was added at the 60 s time point, and a dramatic

redistribution of fluorescent intensities from the membrane to the cytosol was observed ~60 s after ionomycin treatment. In the cytoplasm the pre-treatment signal was ~100 kcps and post treatment the signal was ~700 kcps. At the membrane both signals decreased; for the top membrane the signal dropped from ~200 kcps to ~0 while the bottom membrane signal dropped from ~200 kcps to ~100 kcps. The fit of the z-scan profile to Eq. 6 also recovered the cell's height which destabilized upon treatment with ionomycin. This was particularly evident in cells expressing PH-PLC δ -EGFP where the time trace (Fig. 6B) identified a reduction in cell height of 0.33 μ m after ionomycin treatment. The change in height was calculated by comparing the average height from the interval between 0–60s to the average height in the interval from ~140–200 s. This loss of height was typical for PH-PLC δ -EGFP cells following addition of ionomycin and will be discussed in more detail later. The data further show that the delay between adding ionomycin and fluorescence response cannot be explained by slow mixing of the ionomycin solution with cell medium as demonstrated by the fluorescence intensity trace (Fig. 6C) of Texas Red that was included with the ionomycin solution.

As a control, ionomycin solution was also added to cells expressing EGFP and cells expressing EGFP-H-Ras. The z-scans were fit to a slab model for EGFP and a delta-slab-delta model for EGFP-H-Ras. The fluorescence intensities of EGFP in the cytoplasm as well as the cell height remained approximately constant before and after addition of ionomycin at $t = 60$ s (Fig. 7A). Similarly for EGFP-H-Ras, we observed no significant change in cell height, the fluorescence at the membrane, and the fluorescence in the cytoplasm upon adding ionomycin (Fig. 7B).

We used box and whisker plots to characterize the changes in cell height, cytoplasmic intensity and membrane intensity before and after treatment with ionomycin for all cells measured. The “pre” period represents 60 seconds before the addition of ionomycin. For PH-PLC δ -EGFP, the “post” period was defined to start when the redistribution of the intensity approached its final point and lasted for 60 seconds. PH-PLC δ -EGFP (10 cells) showed the largest change in cell height ($h = h_{post} - h_{pre}$), with a mean loss of -0.37μ m after ionomycin treatment (Fig. 8A). Conversely, both EGFP (10 cells) and EGFP-H-Ras (8 cells) only changed their average cell height slightly (Fig. 8A). The distributions of both are scattered around zero with average values of -0.14μ m for EGFP-H-Ras and -0.10μ m for EGFP. Fig. 8B displays the cytosolic intensity ratio of post- to pre-treatment with ionomycin. Both EGFP and EGFP-H-Ras have ratios close to unity (mean of 1.04 for EGFP and 1.07 for EGFP-H-Ras), which demonstrates the absence of a strong ionomycin-specific effect, in agreement with the data of Fig. 7. The cytosolic intensity ratio of PH-PLC δ -EGFP has a mean value of 5.13 which corresponds to a five-fold increase in the cytosolic intensity following the addition of ionomycin. The membrane intensity ratio of post-ionomycin to pre-ionomycin values is shown in Fig. 8C. Because EGFP-H-Ras has an intensity ratio of ~1 (mean of 0.98), the distribution of proteins at the membrane is essentially not affected by the ionomycin treatment. PH-PLC δ -EGFP has a membrane intensity ratio centered at 0.60 reflecting the redistribution of PH-PLC δ -EGFP proteins from the membrane to the cytoplasm.

Although changes in the EGFP-H-Ras signal with ionomycin treatment were relatively small, we observed a measurable difference in the cytoplasmic ($F_{cyto} = F_{cyto,post} - F_{cyto,pre}$) and membrane-bound ($F_{mem} = F_{mem,pre} - F_{mem,post}$) intensity for each treated cell. The difference F_{mem} was calculated using the average of both membranes. We next compared the changes in the observed cytoplasmic and membrane-associated intensity by graphing the ratio F_{cyto}/F_{mem} for all of the HRas-EGFP cells studied (Fig. 9). The median of the ratio is close to one for the majority of cells (median of 0.58 and mean of 0.89) and is relatively stable considering the small observed signal.

In contrast to HRas-EGFP the redistribution of PLC δ -EGFP from the membrane to the cytoplasm resulted in a large change in the cytoplasmic intensity coupled with a smaller change in the intensity at the membrane (Fig. 6). The ratio of the cytoplasmic to membrane-associated intensity difference (F_{cyto}/F_{mem}) for all of the PH-PLC δ -EGFP cells studied (Fig. 9) varied from ~3 to 15 with a mean of 8.4, which is considerably larger than observed for HRas-EGFP.

As described earlier, the fluorescence intensity is connected to brightness, concentration and PSF volume or area, which leads to $F_{cyto} = \lambda(c_{cyto,post} - c_{cyto,pre})V_{\infty}$ and $F_{mem} = \lambda(\sigma_{mem,pre} - \sigma_{mem,post})A_0$. Thus, the ratio of the intensity difference relates to concentration changes in the cytoplasm and at the membrane along the scan path,

$$\frac{\Delta F_{cyto}}{\Delta F_{mem}} = \frac{c_{cyto,post} - c_{cyto,pre}}{\sigma_{mem,pre} - \sigma_{mem,post}} \frac{V_{\infty}}{A_0}. \quad (8)$$

If we assume that the protein is approximately uniformly distributed inside the cell and at the membrane, then the intensity ratio can be connected to the plasma membrane area S_{mem} and the cell volume V_{cell} . The number of PH-PLC δ -EGFP or EGFP-H-Ras molecules before and after ionomycin treatment has to be conserved, which implies

$$\sigma_{mem,post}S_{mem} + c_{cyto,post}V_{cell} = \sigma_{mem,pre}S_{mem} + c_{cyto,pre}V_{cell}. \quad (9)$$

Inserting Eq. 9 into Eq. 8 leads to

$$\frac{\Delta F_{cyto}}{\Delta F_{mem}} = \frac{S_{mem}}{V_{cell}} \frac{V_{\infty}}{A_0}, \quad (10)$$

The equation states that the intensity ratio is directly related to the ratio of the membrane surface area S_{mem} over the cell volume V_{cell} provided the protein is uniformly distributed. When accounting for the PSF volume and area of the experimental setup ($A_0 = 0.18 \mu\text{m}^2$ and $V_{\infty} = 0.24 \mu\text{m}^3$) the average of F_{cyto}/F_{mem} predicts a surface to volume ratio (S_{mem}/V_{cell}) of $6.7 \mu\text{m}^{-1}$ for PH-PLC δ -EGFP and $0.7 \mu\text{m}^{-1}$ for EGFP-H-Ras. These two predictions differ widely, which will be discussed later in more detail.

Discussion

Distinguishing cytoplasmic and membrane-bound protein populations relies on the accurate breakdown of the z-scan intensity profile into its individual components. A prerequisite for this deconvolution is an accurate PSF model and parameterization. It is crucial to test the selected PSF model on experimental z-scan traces with well-defined geometries. The delta-layer is optimal to test the PSF model, because it directly probes the radially integrated PSF (RIPSF) profile (Fig. 2F). A second important test model is the semi-infinite layer (Fig. 2D). The same PSF parameters that describe the delta layer also need to model the z-scan intensity profile of the semi-infinite layer. Because the semi-infinite layer probes the PSF deeper into the solution than the delta layer, depth-changes in the PSF, such as those caused by spherical aberrations, are easily noticed. There should be no deviation between the model and the experimental intensity profile, at least to a depth that covers the size of the object to be scanned, which in the case of adherent cells is a few micrometers. A PSF model that passes the above tests is viable for z-scan FPD analysis. We previously demonstrated that the modified Gaussian-Lorentzian PSF model [14,15] is suitable for our two-photon instruments and identified the PSF parameters.

Identifying the cytoplasmic and membrane-bound intensities is based on fitting of the z-scan intensity profile. Reliable extraction of the fit parameters requires the correct assignment of measurement uncertainties in addition to selecting an accurate PSF model. We calculated the uncertainty based on shot noise, which resulted in fits with reduced chi-squared values of ~ 1 for the experimental intensity traces. This result implies that shot noise due to the photon detection process is the dominant source of noise of experimental z-scan intensity traces, which is further corroborated by the simulated z-scan intensity profiles that give rise to uncertainties in the fractional membrane intensity that closely match the experimental data (Fig. 4A).

Extracting the intensities from the membrane and cytoplasm relies on the shape of the intensity profile. Thus, the cell at the scan location has to be sufficiently thick to generate a profile with the two membrane intensity peaks readily distinguishable. As a rule of thumb the thickness should be at least twice the axial beam waist z_0 of the PSF, which implies a minimum thickness of $\sim 2 \mu\text{m}$ for our setup. This condition was satisfied for all cell measurements presented in this study. Furthermore, the amplitude of the membrane intensity peaks needs to be strong enough to measurably influence the shape of the intensity profile. While peaks are no longer visible when the membrane intensity fraction falls below 25%, quantitative analysis of the z-scan intensity profile extends to lower membrane intensity fractions. The experiments and simulation identify a practical lower limit for the membrane intensity fractions of ~ 0.1 (Fig. 4A).

The uncertainty in the fit parameters increases rapidly at low membrane intensity fractions. Collecting more photons during the scan reduces the fit uncertainty, but increasing laser power is limited by the onset of photobleaching of the sample, which distorts the intensity profile. Slowing down the z-scan speed would collect more photons, but this approach requires a very stable instrument and may not be an option in many cases. Our instrument requires relatively fast scans to counteract the distorting influence of focal drift.

Measurements over several months identified a maximum drift rate of ~10 nm/s. In this study we collected a complete intensity profile in 5 s (half a scan period), which is sufficiently fast to render the influence of drift negligible. While we observed slow changes in cell height in some cases, the cell thickness at the scan location is usually remarkably constant for prolonged periods of time. A stable cell thickness provides an alternative to improve the signal-to-noise ratio of the measurement that does not rely on a slow scan speed. We demonstrated that summing the signal from repeated, fast z-scans is feasible and results in a lower uncertainty in the fit parameters. Together, these results establish that quantitative z-scan analysis can be performed on a regular microscope without the need for specialized hardware that corrects focus drift.

FPD analysis of the z-scan intensity determines the intensity from each fluorescent layer and the separation between layers. The scanning rate used to collect fluorescence intensity profiles is fast enough that the layer intensity and separation can be applied to time-resolved studies of protein binding to the membrane, as demonstrated by the data in Fig. 6. Furthermore, it is possible to relate intensities to concentration through the use of a conversion factor from an independent control experiment. In our case, we determined the brightness λ_{EGFP} of monomeric EGFP in an independent cell experiment. The protein concentration in the cytoplasm and at the membrane is calculated from the intensities and the brightness as explained in the results section. Because λ_{EGFP} corresponds to the brightness of monomeric EGFP, the calculated concentration is expressed in terms of monomer. In contrast to the z-scan intensity profile, z-scan FFS is a slow technique which is not suited for time-resolved studies because the additional FFS measurements lead to data acquisition times approaching 100 s. Thus, z-scan FFS measurements only have an advantage over z-scan FPD, if the oligomeric state of proteins at the plasma membrane and in the cytoplasm needs to be identified.

This study applied z-scan FPD with a time resolution of 5 s to quantify the redistribution of PH-PLC δ -EGFP from the plasma membrane to the cytoplasm upon ionomycin treatment. Since the time scale of the cellular response to signaling events typically is longer than a minute [24], a resolution of 5 s should be sufficient in most cases. We monitored the appearance of Texas Red fluorescence to account for the variability in the mixing time upon adding ionomycin solution (Fig. 6), which served to identify the exact time point of ionomycin delivery to the cell. The experiment recorded a time delay between ionomycin delivery and the onset of the fluorescence intensity redistribution (Fig. 6). The delay of ~30s is consistent with studies of Ca²⁺ cell signaling which showed that Ca²⁺ reached maximum levels 30 – 60s after ionomycin treatment [21,25,26].

The membrane translocation kinetics of peripheral membrane proteins like PH-PLC δ -EGFP have been studied by fluorescence imaging methods. Initial studies used confocal imaging to show the redistribution of PH-PLC δ -EGFP from the membrane to the cytoplasm after ionomycin treatment or PFA receptor stimulation [10,11]. Both studies used the intensity profile from a line segment of the image to quantify the relative change in fluorescence around the membrane region over time. While this captured the kinetics of the process, the profile itself provides no direct measure for separating cytoplasmic and membrane-bound components. Loew and colleagues noted another problem with intensity profiles linked to

membrane movement. They imaged PH-PLC δ -EGFP and observed bradykinin stimulation-induced cell shape changes and membrane movement [12]. To address this issue, a region of interest including membrane and cytoplasm was selected, followed by a thresholding step to segment the membrane region. The average intensity above the threshold was used to estimate the intensity at the membrane [12]. While this approach was judged to avoid issues associated with membrane movement, the local cell geometry within the region of interest and a precise PSF model were not taken into account, which prevented a truly quantitative separation of membrane-associated and cytoplasmic fluorescence. Z-scan FPD overcomes these shortcomings and achieves quantitative separation of the intensity profile by analytically accounting for cell geometry and PSF shape.

The inherent quantitative nature of z-scan FPD analysis allowed us to measure membrane movement that had been mentioned as a concern but was not quantified [12]. PH-PLC δ -EGFP showed a significant reduction in cell height upon ionomycin treatment (Fig. 8A). Control experiments on cells expressing EGFP and EGFP-H-Ras revealed a negligible effect of ionomycin on cell height. Because PH-PLC δ -EGFP lacks enzymatic activity of its own, the results imply that the observed change in cell height was specific to the dissociation of PH-PLC δ -EGFP from the plasma membrane and that the interaction of the peripheral membrane protein with the lipid had an influence on the plasma membrane and the cell shape. This observation agrees with other studies that have pointed out that protein-membrane interactions can change properties of the membrane itself [4–7], as well as, modulate the adhesion strength between the cytoskeleton and membrane lipids [27]. For example, PH-PLC δ -EGFP has been shown to induce positive membrane curvature in model membranes when it binds to PtdIns(4,5)P₂ lipids [28]. While the previous study was performed *ex situ*, our results demonstrate that PH-PLC δ -EGFP binding also affects the membrane inside a living cell. In addition, we observed a slight asymmetry in the response of the top and bottom membrane upon ionomycin treatment. While the fluorescence intensity from PH-PLC δ -EGFP associated with either membrane was approximately identical before treatment, the top membrane showed consistently a more pronounced decrease in fluorescent intensity than the bottom membrane following ionomycin treatment (Fig. 6A). The cause for this difference is currently not known and will require further study. However, since the rigidity of the glass substrate can alter the bottom membrane's properties [29,30], the membrane-glass attachment might contribute to the observed effect. Z-scan FPD provides the means to quantitatively study differences at the top and bottom membrane, which is not readily accessible by other fluorescence techniques. The influence of substrate rigidity can be explored in future experiments by coating the glass coverslips in order to change the substrate stiffness.

An estimate of the surface area to volume (SA/V) ratio of U2OS cells based on a simple model of the cell as a cylinder with a cross-sectional area of $\sim 400 \mu\text{m}^2$ and a volume of $\sim 1 \text{ pl}$ [31] leads to a value on the order of $1 \mu\text{m}^{-1}$. This estimate predicts that changes in fluorescence at the plasma membrane and in the cytoplasm due to translocation results in a ratio $F_{\text{cyto}}/F_{\text{mem}}$ of ~ 1 (Eq. 10) provided that the concentration at the membrane and in the cytoplasm are uniform. As mentioned above, the SA/V ratio for EGFP-H-Ras is $0.7 \mu\text{m}^{-1}$ which is in good agreement with the above estimate, but for PH-PLC δ -EGFP we

arrived at a ratio ($SA/V=6.7 \mu\text{m}^{-1}$) that is inconsistent with the model. The ratio is significantly larger than the expected value which indicates the appearance of more protein in the cytoplasm than is expected from the loss at the plasma membrane. This outcome could be achieved by the additional release of PH-PLC δ -EGFP from internal membranes like the golgi, but studies have found very little association of PH-PLC δ -EGFP with internal membranes [32,33]. Another source that could account for the large ratio is the presence of plasma membrane areas outside the z-scan location that carry a higher protein concentration. It has been shown that PtdIns(4,5)P₂ and PH-PLCdelta-EGFP are more highly concentrated in membrane ruffles [32,34–37] compared to other regions of the membrane. The ruffles of U2OS cells are located closer to the periphery of the cell, a region that was not sampled in our study, because it is too thin for z-scan FPD analysis. Thus, a likely explanation for the high ratio is the release of additional PH-PLC δ -EGFP located at membrane ruffles.

In summary, z-scan FPD analysis introduces a method to decompose the fluorescence contributions from the cytoplasm and membrane of a peripheral membrane protein in the living cell. Only three elements are required, the fluorescent intensity profile along the axial dimension of the cell, a well characterized point spread function, and a model of the cell geometry. Intensities both at the membrane and in the cytoplasm are readily converted into concentrations by including a brightness calibration measurement taken in EGFP expressing cells. The quantitative nature of z-scan FPD, as demonstrated by our results, opens new opportunities for investigating peripheral membrane proteins in cells. It will be interesting to explore combining this method with lateral scanning in future development work. Such an extension of the technique would allow probing lateral heterogeneity of protein density at the membrane, such as the presence of punctate structures, while still retaining the ability to quantitatively distinguish fluorescent intensities contributions from cytoplasmic and membrane layers.

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Figure 1.

Concept of z-scanning. The two-photon excitation spot is scanned along the z-axis of a cell expressing a fluorescently labeled peripheral membrane protein which results in a z-dependent fluorescence intensity profile.

Figure 2.

Z-dependent concentration function for a slab (A), semi-infinite (C), and delta (E) layer. The lower panels show an experimental intensity profile $F_{\text{exp}}(z)$ (solid line) and fit to a model intensity profile $F_{\text{mod}}(z)$ (shaded line) for each concentration function. (B) The intensity profile $F_{\text{exp}}(z)$ of a fluorescent cell is described by the slab model ($\chi_r^2=1.2$). The maximum intensity F_{max} is less than the intensity F_{∞} for the infinite sample. (D) Intensity profile of a fluorescent solution matches the semi-infinite model ($\chi_r^2=1.1$). (F) Intensity profile of thin fibronectin layer is in good agreement with $F_{\text{mod}}(z)$ for a delta layer ($\chi_r^2=1.0$).

Figure 3.

The delta-slab-delta model. (A) Concentration profile. (B) Z-scan intensity profile for EGFP-H-Ras (solid line) and fit (shaded line) to Eq. 6 ($f_B = 0.92, f_T = 0.95, \chi_r^2 = 1.2$) with normalized residuals in lower panel. (Dotted-dashed line) Contribution from the cytoplasmic layer; (dotted lines) contribution from the membrane layers. (C) and (D) Intensity profile of cells expressing EGFP-H-Ras and mCherry fit to delta-slab-delta model (χ_r^2 of 1.3 and 1.1).

Figure 4.

Resolvability of membrane intensity fractions f_M from z-scan intensity profiles. (A) Relative error (stars) versus the membrane intensity fraction f_M . Each data point was determined from fits of 10 consecutive scans. All intensity profiles were taken from cells with cytoplasmic intensities $F_{\text{cyto},\infty}$ between 100 and 1000 kcps. Relative error in f_M from simulated intensity profiles ($n=1000$) for $F_{\text{cyto},\infty} = 10$ kcps (squares), 100 kcps (triangles), 1000 kcps (diamonds). (Inset) Relative error versus mean of z-scan intensity profiles from cells with $F_{\text{cyto},\infty}$ between 11 and 16 kcps. Relative error from individual scans (open circles) is

reduced by summing groups of 10 scan profiles (solid circles). (B) Relative error in f_M as a function of the number of summed intensity profiles for one cell (squares) from the inset.

Figure 5. Repeated z-scans performed at the same x-y position in an U2OS cell expressing EGFP-H-Ras are fit by Eq 6. (A) Top (black circles) and bottom (shaded circles) membrane position as a function of time show parallel movement with $\sim 0.5 \mu\text{m}$ of z-drift over a period of 12 minutes. (B) Cell thickness (solid line) stays approximately constant. (C) Top (dashed line) and bottom (solid line) membrane intensity as a function of time is constant ($F_{\text{BM}} = 88.8 \pm 3.1 \text{ kcps}$, $F_{\text{TM}} = 108.4 \pm 3.1 \text{ kcps}$). (D) Cytoplasmic intensity (dot-dashed line) remains stable ($F_{\text{cyto}} = 16.2 \pm 0.9 \text{ kcps}$).

Figure 6.

Kinetics from repeated z-scans of a U2OS cell expressing PH-PLC δ -EGFP. (A) Top membrane (dashed line), bottom membrane (solid line), and cytoplasmic (dotted-dashed line) intensity as a function of time. Ionomycin solution was added at 60 s. (B) Cell height (solid line) as a function of time. Average height from 0–60 s (dotted line) and from 140–200 s (dotted-dashed line) shows a reduction of 0.34 μm . (C) Intensity time trace of Texas Red from the ionomycin solution.

Figure 7.

Kinetics from repeated z-scans of U2OS cells with ionomycin solution added at 60 s. (A) EGFP expressing cell with cytoplasmic intensity (upper panel) and cell height (lower panel) as a function of time. (B) EGFP-H-Ras expressing cell with intensity from top membrane (dashed line), bottom membrane (solid line), and cytoplasm (dotted-dashed line). Lower panel shows cell height.

Figure 8.

Box plots of changes in cell height and intensity between pre- and post-ionomycin treatment of U2OS cells expressing PH-PLC δ -EGFP ($n = 10$), EGFP ($n = 10$), or EGFP-H-Ras ($n = 8$). (A) Difference in cell height $h = h_{\text{pre}} - h_{\text{post}}$ (mean of $-0.37 \mu\text{m}$ for PH-PLC δ -EGFP, $-0.10 \mu\text{m}$ for EGFP, $-0.14 \mu\text{m}$ for EGFP-H-Ras). (B) Cytoplasmic intensity ratio ($F_{\text{post}}/F_{\text{pre}}$) with a mean values of 5.13 for PH-PLC δ -EGFP, 1.04 for EGFP, 1.07 for EGFP-H-Ras. (C) Membrane intensity ratio ($F_{\text{post}}/F_{\text{pre}}$) with mean values of 0.60 for PH-PLC δ -EGFP and 0.98 for EGFP-H-Ras.

Figure 9.

Box plots of the ratio of $F_{\text{cyto},\text{post}} - F_{\text{cyto},\text{pre}}$ and $F_{\text{mem},\text{pre}} - F_{\text{mem},\text{post}}$ for ionomycin treated U2OS cells expressing PH-PLC δ -EGFP ($n = 10$) and EGFP-H-Ras ($n = 8$).