

Video Article

Determination of Plasma Membrane Partitioning for Peripherally-associated Proteins

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URL: <https://www.jove.com/video/57837>

DOI: [doi:10.3791/57837](https://doi.org/10.3791/57837)

Keywords: Biology, Issue 136, Peripherally-associated plasma membrane protein, membrane affinity, membrane partitioning, confocal microscopy, ImageJ, R-project, FM 4-64, deconvolution

Date Published: 6/15/2018

Citation: Vosolsobě, S., Schwarzerová, K., Petrášek, J. Determination of Plasma Membrane Partitioning for Peripherally-associated Proteins. *J. Vis. Exp.* (136), e57837, doi:10.3791/57837 (2018).

Abstract

This method provides a fast approach for the determination of plasma membrane partitioning of any fluorescently-tagged peripherally-associated protein using the profiles of fluorescence intensity across the plasma membrane. Measured fluorescence profiles are fitted by a model for membrane and cytoplasm fluorescence distribution along a line applied perpendicularly to the cell periphery. This model is constructed from the fluorescence intensity values in reference cells expressing a fluorescently-tagged marker for cytoplasm and with FM 4-64-labeled plasma membrane. The method can be applied to various cell types and organisms; however, only plasma membranes of non-neighboring cells can be evaluated. This fast microscopy-based method is suitable for experiments, where subtle and dynamic changes of plasma membrane-associated markers are expected and need to be quantified, e.g., in the analysis of mutant versions of proteins, inhibitor treatments, and signal transduction observations. The method is implemented in a multi-platform R package that is coupled with an ImageJ macro that serves as a user-friendly interface.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57837/>

Introduction

Peripherally-associated plasma-membrane proteins are the key components of cell signaling pathways. One of their fundamental roles is their transient plasma membrane association and dissociation, which is important for the signal transduction between plasma membrane and cytoplasm. Peripherally-associated plasma membrane proteins can be attached on plasma membrane by lipid anchors (N-myristoylation, S-acylation, or prenylation) or by lipid binding domains (interacting with phosphatidylinositol phosphates, phosphatidic acid, etc.).

Plasma-membrane binding properties of these proteins can be examined *in vivo*, e.g., when a fluorescently-tagged protein is modified by a site-directed mutagenesis of key amino acids, or when it is treated with various inhibitors affecting lipid signaling. The distributions of peripheral plasma membrane proteins are mostly being evaluated qualitatively, especially in cases, when protein re-distribution is obvious. The presented method is optimal for situations when protein re-distribution is only partial and quantitative evaluation is necessary. A frequently used approach of when plasma membrane association is estimated from confocal laser scanning microscopy images as a ratio of fluorescence intensities at the plasma-membrane and in the cytoplasm^{1,2}, is simple, but not accurate. Fluorescence intensities at the plasma membrane reflect a superposition of the plasma-membrane and cytoplasm signal due to the light diffraction characteristic for the particular fluorescence microscopy technique and optical elements used³. Consequently, the cytoplasmic signal is included also in the membrane region. For this reason, FM 4-64 staining pattern cannot be used as a mask for a membrane signal selection⁴. Furthermore, simple measurements of membrane signal at the position defined by the FM 4-64 staining maximum always systematically overestimate the real plasma-membrane signal of peripherally-associated plasma-membrane protein due to the superposition of the membrane and cytoplasmic compound. The maximum of observed signals for fluorescently-tagged peripherally-associated proteins also does not co-localize with the maximum of the plasma membrane marker (i.e., FM 4-64 styryl dye), but is shifted towards the cytoplasm. Another limitation is based on the fact that the FM 4-64 emission peak is wider in comparison with the emission peaks for green fluorescent proteins such as GFP due to the wavelength-dependency of light diffraction³.

In the method described here, the tagged protein signal is fitted by two empirical functions describing a hypothetical distribution of the plasma membrane and cytoplasm signal, respectively. This signal decomposition is applied to linear fluorescence profiles that are applied to the cell surface perpendicularly to the plasma membrane in source images, which are regular, two-channel confocal sections of fluorescently-tagged protein expressing cells labeled with FM 4-64 dye.

The first function used for fitting describes a diffraction of a cytoplasm signal on the cell edge. It is obtained from previously acquired fluorescence profiles that were measured in cells expressing a cytoplasm protein marker tagged by the same chromophore as the plasma membrane peripherally-associated protein of interest. The second function describing a diffraction of a plasma-membrane signal is derived from the fluorescence of FM 4-64. This signal is firstly approximated by a Gaussian function that is being used for an approximate modeling of light

diffraction of a point source. Secondly, this model, valid for red FM 4-64 emission, is mathematically transformed to the form that is relevant for an emission wavelength of the chromophore used for the tagging of peripherally-associated proteins of interest at the plasma membrane. Both functions are normalized by the maximal intensity and by the mean from 10% of the highest values for FM 4-64 signal and cytoplasmic protein signal, respectively. By this signal decomposition (non-linear least square fitting method), the ratio of the plasma membrane and the cytoplasm fraction of the examined protein can be estimated easily and accurately. The real physical dimension of computed partitioning coefficient is in the range of micrometer, because cytoplasmic volume concentration is compared with surface concentration on the plasma membrane. It defines the distance from the plasma membrane to the cytoplasm, within which the same amount of proteins is localized as in the adjacent area of the plasma membrane. This value is equivalent to the partitioning coefficient K_2 introduced previously⁵. The method is very quick, requiring only single confocal sections acquired using routine confocal laser scanning microscope, and it is not computationally demanding. The analysis core has been implemented in a portable R package and an additional ImageJ macro was written to provide graphical user interface to run the analysis from the intuitive dialogs. Software and more detailed description of the method (published previously⁶) can be found at <http://kfrserver.natur.cuni.cz/lide/vosolsob/Peripheral/>.

The method is suitable for isolated cells, protoplasts, and tissues, where the plasma membrane of individual cells is clearly distinguishable, expressing a fluorescently-tagged construct of examined peripherally-associated protein. A chromophore compatible with FM 4-64 staining must be used. FM 4-64 emits red fluorescence; therefore, examined protein can be tagged by a fluorescence protein with blue, green, or yellow emission (e.g., GFP, CFP, YFP). Stable transformation of biological material is recommended because it enables less artificial and more reproducible observations of protein distribution. It is necessary that the examined protein has a relatively homogeneous cytoplasmic distribution. The localization of a protein in the endoplasmic reticulum or another intracellular membrane compartment can produce artificial results.

Additionally, the same biological material expressing a cytoplasmic marker must be used for the comparison. Cells can be transformed by a free chromophore (the same as used for peripheral protein tagging, e.g., free GFP) or by tagged protein of interest with abolished membrane binding capacity. Membrane binding capacity can be abolished, for example, by trimming of the membrane-binding domain or by site-directed mutagenesis of key amino acid residues (e.g., sites for N-myristoylation, S-acylation, or prenylation, etc.).

For confocal scanning microscopy, cells must be labeled by a membrane marker like FM 4-64 dye. If FM 4-64 staining is not suitable for the studied material (due to interfering autofluorescence, poor dye penetration, etc.), the plasma membrane can be labeled, for example, by integral plasma membrane protein tagged to an appropriate chromophore (mCherry, RFP, etc.). It is essential that the marker has negligible localization in the intracellular membrane compartments (endomembranes).

If working with fixed samples and antibodies, fixable analogue FM 4-64FX or plasma membrane labeling by antibody against an appropriate target can be used. In this case, it is essential to evaluate results very carefully because fixation procedures can lead to selective loss of proteins from both the cytoplasm and the plasma membrane.

Protocol

1. Preparation of Biological Material

1. Prepare biological material expressing the fluorescently tagged protein of interest, as well as a cytoplasmic marker. Follow the procedures mentioned in the Introduction.

2. Confocal Laser Scanning Microscopy

1. Stain the material prepared in section 1 with FM 4-64 dye⁷.
 1. Apply a staining protocol that is appropriate for the studied material. For tobacco BY-2 cells, stain 200 μ L of cell suspension with 0.2 μ L of 10 mM FM 4-64 solution in dimethylsulfoxide.
Note: The typical staining concentration of FM 4-64 dye is about 10 μ M using 10 mM stock solution in dimethylsulfoxide. Use the lowest possible concentration of FM 4-64 for proper staining and do not incubate cells on ice (FM 4-64 and low temperature can affect plasma membrane properties). Continue with observation using confocal microscopy immediately. The interval between the staining and the image acquisition should not be longer than 10 min; otherwise, endocytosis of the dye will affect the FM 4-64 staining.
 2. Process multiple samples consecutively, and do not stain all the samples at the beginning of the experiment.
2. Capture one equatorial confocal section per one cell.
 1. Set a sequential two-channel scanning for the chromophore used as the protein tag (must be in the first channel) and FM 4-64 (must be in the second channel). Set a higher optical resolution (10–20 px/ μ m). An example set-up: 63X oil immersion objective, NA 1.3, image size 1,024 x 1,024 px. Ensure that the plasma membrane plane is perpendicular to the confocal-section plane in the acquired images.
 2. Capture at least 20 cells per sample to have enough data for the statistic evaluation (depends on the signal variability in processed material).

3. Required Software Installation

1. Install Fiji ImageJ distribution⁸ and the required macro peripheral.
 1. Download software from the site <https://fiji.sc/#download>, and install them by unpacking.
 2. Start Fiji by double-clicking on the "ImageJ(.exe)" file in the unpacked "Fiji.app" directory.
 3. Download a macro "peripheral" from the following site:
http://kfrserver.natur.cuni.cz/lide/vosolsob/Peripheral/source/Peripheral_.ijm.

4. In Fiji, select **Plugins | Macros | Install...** in the main menu and specify the path to the downloaded file "Peripheral_.ijm."
Note: Two new tools of the installed macro "peripheral" will appear in the Fiji toolbar: "Take profile Tool" and "Peripheral Protein Menu."
2. Install the R-project software⁹ and required packages.
 1. Follow the instructions on the site <https://cloud.r-project.org/> for R-project installation.
Note: The overall analysis will be performed in Fiji. The R software will be called only internally by the Fiji macro "peripheral" during computation.
 2. Run R GUI, select **Packages | Install package(s)...** in the main menu, and specify the nearest repository and package "**ggplot2**" for installation.
Note: Alternatively, type to the R command-line: `install.packages("ggplot2")`.
 3. Download an R package peripheral from the destination: http://kfrserver.natur.cuni.cz/lide/vosolsob/Peripheral/source/peripheral_latest.tar.gz. Install them by selecting **Packages | Install package(s) from local files....**
Note: Alternatively, type to the R command-line this command only:
`install.packages("http://kfrserver.natur.cuni.cz/lide/vosolsob/Peripheral/source/peripheral_latest.tar.gz", repo=NULL, type="source")`.

4. Image Analysis in Fiji

1. Process the confocal images of the cytoplasmic marker.
 1. Import the images to Fiji using **Plugins | Bio-Formats | Bio-Formats Importer** from the Fiji menu with default settings.
 2. Check if the Bio-Formats Importer properly recognized the image calibration. The picture dimension shown in the upper left information field image window must be equal to the original picture dimensions.
 3. Treat improperly calibrated images with the incorrect dimensions individually, e.g., by setting the correct parameters in the dialog window **Analyze | Set Scale...** from the Fiji menu: fill the image width in pixels into the **Distance in pixels** field and the real width in the appropriate length unit into the **Known distance** field).
Note: If the Leica LCS LEI files are improperly calibrated, follow step 4.2.1.
2. Run the **Import options macro** for an analysis set-up. Activate the macro by one of three different ways: select **Plugins | Macros | Import options [f1]** in the main Fiji menu, select the same item after clicking the menu tool **Peripheral Protein Menu**, or press the keyboard shortcut F1. Set the parameter in the invoked macro dialog window.
 1. In case of the Leica LCS LEI format, solve **improper calibration** by activating **Leica.lei images** check-box. This option properly imports the image dimensions from the file with the "lei" extension.
 2. Define the appropriate value in the field **Gaussian blur radius (px)**. This influences picture smoothing and noise reduction. A low value (1 px) is sufficient and does not cause any image information loss.
 3. Set a value in the field **Profile line width (px)**. A thicker line causes higher smoothing of the profile curves. A default value 10 px is recommended.
 4. Set an image title parsing by the definition of **"Sample delimiters"** and **"Exported items."**
Note: An image title (filename without extension, e.g.: "Experiment-1_line-02_cell-11") will be split around all the characters listed in the field **"Sample delimiters"** (e.g.: "-_") into a set of items ("Experiment", "1", "line", "02", "cell", and "11") that can be used as **grouping factors** (sample, cell, line, or treatment identity) in the following analysis. Define which items will be used by typing their sequence number ("0" for the first item) into the field **"Exported items."** Use space-delimited format, e.g., "3 5." In this example, items "02" and "11" will be referred to as grouping factors named "Fac_0" and "Fac_1." Additionally, the identity of each measurement will be referred to as "I" factor.
 5. Alternatively, keep the **Sample delimiters** empty and enter 0 into the **Exported items** if the full image title may be retained.
 6. On the Windows operation system, check if the path to the R software will be correctly auto-detected in the field **Path to R.exe**. Otherwise, specify the full path to the R.exe file in the system (e.g., "C:\Program Files\R\bin\R.exe").
 7. Click **OK**. Check the title parsing in the next dialog window and click **OK** or **Back** to reset.
Note: All pictures will be automatically smoothed and eventually recalibrated. A list of all exported grouping factors will be displayed in the Results window.
3. Perform a linear selection across the plasma membrane region in the processed images and measure the fluorescence profiles.
 1. Select the Fiji tool **Straight line** from the Fiji toolbar. Click the image and drag a line across the plasma membrane region. The line must start in the extracellular space and should be perpendicular to the plasma membrane. Choose regions with a thicker and more homogeneous layer of cortical cytoplasm. The optimal length of the linear selection is 5 - 10 μm .
 2. Run the **Take profile macro** by analogical method as in the step 4.2 (shortcut "x") or by clicking **Take profile Tool** in the Fiji toolbar. The automatic measurement of fluorescence profiles in both channels will be performed and data will be displayed in the Results window.
 3. If using the "x" key is considered user-unfriendly, open the macro source file **Peripheral_.ijm** in the text editor, replace the "x" symbol in the line 'macro "Take profile [x]" {' with a more favorable symbol (that is not used in the Fiji environment as an active keyboard shortcut) and save the file. Reinstall the macro (step 3.1.4) and start the analysis from the image import.
 4. According to individual signal variability, take representative numbers of the profiles for each cell.
 5. Save the data via **File | Save As....** Always use the "csv" extension; it is necessary for proper data format.
4. Run the **Plot profile data macro** (short-cut F5) for graphical visualization of the measured profiles. Set the parameter in the invoked macro dialog window.
 1. Keep the check-box **Use filtering for input data?** unselected.
 2. Specify if the plot should be created from **recent data** in the Result window, from a **single CSV file** or **from all CSV files** in a specified directory by clicking the appropriate radio button.
Note: If the plot is created from recent results, data will be saved first (**Save As...** dialog will be automatically activated).

3. Specify which **grouping factors** will be used for the plotting by selecting the appropriate check-boxes. Select the factor "i." if all profiles should be displayed in unique plots. Keep the check-boxes unselected, if all data should be plotted in a single plot.
CAUTION: Selecting of a grouping factor that does not exist in the processed results causes error during the plot creation.
4. Specify the **filename** when saving a plot in the field **Output file prefix** and define the plot **image dimensions** in fields **Plot high** and **Plot width**.
Note: The resulting plot will be saved in a source data directory as a PNG file.
5. Select the **Pdf output?** check-box if an additional PDF output may be produced.
6. Click **OK**. Specify the path for saving the results or eventually for data import in the next dialog window. Confirm analysis by clicking **OK** in the dialog windows showing the performed R code.
Note: The plot will be opened in a new image window and the R analysis output will be listed in text window.
5. Run the **Profile filtering setup macro** (short-cut F2) if some plotted profiles have excessive signals in the extracellular space (for the tagged protein) or in the intracellular space (for the FM 4-64 signal). Follow by setting the parameters in the invoked macro dialog window.
Note: Filtering enables the removal of poor profiles according to the maximum allowed intensity threshold at specified x-coordinates.
 1. For each channel, set the appropriate intensity threshold in the field **Remove measurements with an excessive extracellular (intracellular) signal**. Specify the region where the intensity threshold will be applied in the field **at x-coordinates lower (greater) than**.
 2. Run the **Plot profile data macro** (step 4.4) again with the check-box **Use filtering for input data?** selected; ensure that all aberrant profiles were successfully removed. Otherwise, improve the filtering parameters (step 4.5.1).
6. Run the **Create model macro** (short-cut F3) to create a model of the plasma membrane and the cytoplasm fluorescence distribution based on the calibration data. Follow by setting the parameters in the invoked macro dialog window.
 1. Specify if the input data should be filtered (step 4.5) by selecting the **Use filtering for input data?** check-box.
 2. Specify the **source data** analogically as in step 4.4.2.
 3. Specify an **interval** at which the model will be predicted by definition of "start," "end," and "step" values in μm .
Note: All profile measurements should be longer than the specified interval.
 4. Specify the **wavelengths of fluorescence** excitation and emission maxima of the chromophores used, in the appropriate fields. Set the defaults for GFP and FM 4-64 combination.
Note: "GFP" indicates the chromophore used for the protein tagging (GFP, CFP, YFP, etc.), and "FM4" indicates the chromophore used for a plasma membrane staining (typically FM 4-64).
 5. Specify the **Output file prefix** field. The prefix will be used for saving a resulting model as the RData file to a source data directory.
 6. Select the check-box **Plot model?** If the graphical output is required. The plot will be saved as the PNG and also as the PDF file if the **Pdf output?** check-box is selected.
 7. Click **OK**. Specify the input or output paths in the next dialog windows and confirm the analysis by clicking **OK** in the dialog window showing the performed R code.
Note: The plot of the modeled cytoplasmic and membrane compound of the peripherally-associated protein fluorescence will be shown in the new image window, and the R analysis output will be listed in the text window.
7. Process the images of the cells expressing the protein of interest.
 1. Import the images analogically as in step 4.1.
 2. Set up the analysis analogically as in step 4.2. The smoothing and line width should be identical.
 3. Measure the profiles analogically as in step 4.3.
 4. Check the accuracy of the profiles by plotting (step 4.4) and set the filtering if desired (step 4.5).
8. Run the **Calculate distribution macro** (short-cut F4) for the calculation of a protein partitioning between the plasma membrane and in the cytoplasm. Follow the parameter setting in the invoked macro dialog window.
 1. Analogically with the previous step (step 4.4), specify the data source, filtering, and filename prefix.
 2. Specify if the **model** for protein distribution calculation will be loaded from the **last model computation** in the recent instance of the "Peripheral" macro on Fiji (select check-box **Recent results**), or from the **RData file** (select check-box **"From file"**).
 3. Keep the check-box **Remove results with residual variability greater than** activated if a result filtering is desired. Specify the threshold of the maximum allowed residual variability that can be unexplained by the signal decomposition of the individual profile measurement. For example: A value of 0.200 indicates that all measurement with unexplained variability higher than 20% of the total variability of the fluorescence intensity in the profile will be discarded.
 4. Select **sample grouping factors**, which may be used for box-plot creating (step 4.4).
 5. Activate the check-box **Pdf output?** for saving the box-plot as the PDF file.
 6. Click **OK**, specify the input or output paths, and confirm the analysis by clicking **OK** in the dialog window showing the performed R code.
Note: The box-plot of peripherally-associated protein partitioning between the plasma membrane and the cytoplasm will be shown in a new image window, and the R analysis output will be listed in text window.
 7. For external processing, save the results displayed in the **Results** window via **File | Save As...** in the window menu.

Representative Results

DREPP¹⁰ is a plant-specific peripherally-associated plasma-membrane protein that is associated with the plasma membrane via an N-myristoylation and an electrostatic interaction with phosphatidylinositol phosphates^{11,12}. DREPP was described as a component of calcium-signaling machinery in the plant cell and also interacts with the cytoskeleton^{13,14}. In the presented experiment, the wild-type tobacco DREPP2 and its non-myristoylated mutant version (Gly2 substituted by Ala) were GFP-tagged⁵ and expressed in tobacco BY-2 suspension cells¹⁵ under the CaMV 35S promoter¹⁶. The plasma membrane partitioning of these proteins were measured in 3-day-old (3 days after dilution), FM 4-64-labeled (8 μ M) cell cultures⁶ with (Figure 1A) and without (Figure 1B) the addition of phosphoinositide 3-kinase inhibitor Wortmannin (10 μ M)¹⁷, according to the protocol described above. The trimmed version DREPP2(Δ 1-23) lacking the plasma membrane binding domain⁶ was used as a cytoplasm marker for the fluorescence distribution model construction (Figure 1C).

Computed data were square-root transformed (the positive value corresponding to the lowest negative value was added to all data to retrieve only positive data), and data were tested by two-way ANOVA in R⁹. The effects of the mutation and inhibitor treatment were highly significant ($p < 2.2 \times 10^{-16}$ ***). All groups were compared by Tukey HSD test; all groups differed significantly with the exception of DREPP2 treated by Wortmannin and non-treated DREPP2(G2A) (Figure 1D).

These results clearly show that the plasma-membrane association of tobacco DREPP2 protein is the result of a N-myristoylation and electrostatic interaction, which are functioning co-operatively. Only the mutual effect of the N-myristoylation site mutation and Wortmannin treatment caused a full dissociation of the DREPP2 protein from the plasma membrane. These results are in accordance with previously published data⁶.

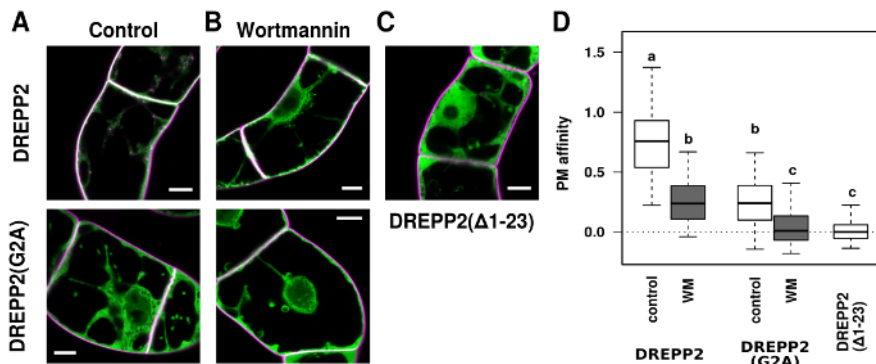


Figure 1: Effect of the mutation in the N-myristoylation site and Wortmannin treatment on the plasma membrane partitioning of peripherally-associated plasma-membrane protein DREPP, which is involved in a calcium signaling pathway in the plant cell. (A) Medial confocal sections of tobacco BY-2 cells expressing the wild type form DREPP2-GFP and the mutant form DREPP2(Gly2Ala)-GFP (green channel). Cells were labeled with FM 4-64 dye (magenta channel), scale bar 10 μ m. **(B)** The same cell line was treated by 10 μ M Wortmannin (WM). **(C)** Cells expressing cytoplasmic marker DREPP2(Δ 1-23). **(D)** The estimated plasma membrane partitioning for all samples. The significance of both effects was tested by two-way ANOVA ($p < 2.2 \times 10^{-16}$ *** in both cases; the original data were square root-transformed). Letters indicate groups that are not significantly different from each other as determined by the Tukey HSD test. Whiskers of the box-plots indicate 95% confidence interval. [Please click here to view a larger version of this figure.](#)

Discussion

The method described here generates a more accurate estimation of plasma membrane partitioning for peripherally associated proteins compared to other approaches based on measuring fluorescence intensities⁵. The major improvement of this method is that it takes into account the light diffraction and superposition of the plasma-membrane and the cytoplasmic signals. Although these method results are in correlation with results of a simple method based on the comparison of fluorescence intensity at the membrane position with the average cytoplasmic signal (as shown previously⁶), the major benefit of this novel method is the determination of the residual variability (unexplained by signal decomposition) that allows the estimation of the relevancy of the results, especially where the plasma membrane signal is lower than the cytoplasmic signal. The described method is also more robust to the signal noise because the computation of protein partitioning is not based on only one point.

The analysis requires only single two-channel confocal images. In contrast to FRAP approaches¹⁸ based on measurements of protein diffusion dynamics in longer time windows, the described method is more applicable for dynamic *in vivo* approaches, when fast image acquisition is a critical requirement (e.g., signal transduction explorations, inhibitory assays). The method is suitable for quickly obtaining large amounts of data that are sufficient for statistical evaluation.

The method is limited to only a single membrane. The analysis of signals from two closely adjacent membranes of neighboring cells is currently not supported. In this case, the signal fitting is more demanding with a higher risk of artifacts.

However, the analysis was originally designed for plant suspension cells¹⁵, and it may be applied for analyses of other cell types as well. Pollen tubes and root hairs represent potentially very good targets of this method in plant biology. External membranes of plant epidermal cells can be analyzed after previous verification that the cell walls are not labeled by FM 4-64 and do not exhibit interfering autofluorescence. Protist cells that are free of interfering autofluorescence, yeast cells, fungal cells, as well as animal cells with smooth plasma membrane may be analyzed using the described method; however, for animal cells the analysis of protein distribution on the leading edge of fibroblast cells or on the brush

border of epithelial cells cannot be possible. Due to the flexible analysis settings, FM 4-64 staining may be replaced by other plasma membrane visualization approaches, such as fluorescently tagged proteins. With caution, the method can be used for fixed cells.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This project was supported by NPU I, LO1417 (Ministry of Education, Youth and Sports of the Czech Republic).

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