

Fluorescence Recovery after Photobleaching (FRAP) Assay to Measure the Dynamics of Fluorescence Tagged Proteins in Endoplasmic Reticulum Membranes of Plant Cells

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[Abstract] In this protocol, we used fluorescence recovery after photobleaching (FRAP) to measure the influence that some mutations and drug treatment have on mobility of a green fluorescent protein (GFP)-fused viral transmembrane protein into endoplasmic reticulum membranes (Serra-Soriano *et al.*, 2014). The proteins of interest were transiently expressed in *Nicotiana benthamiana* (*N. benthamiana*) epidermic cells by agro-infiltration. To minimize transient overexpression artifacts, fluorescence intensity values were gathered at 36 hpi using an inverted Zeiss LSM 780 confocal microscope. Only epidermic cells showing moderated expression levels and homogenous distribution through the ER of the GFP-tagged proteins were used for further experiments. To examine the role of actin polymerization in the mobilization of GFP-tagged proteins, we pretreated tissue samples either with latrunculin B, an inhibitor of actin polymerization, or with DMSO as control. The generated fluorescence recovery curves were used to obtain the percentage of maximum fluorescence recovery (MFR), which corresponds to the mobile fraction, and the half-time of maximum recovery ($t_{1/2}$) values.

Materials and Reagents

1. 3-4 weeks old *Nicotiana benthamiana* plants
2. *Agrobacterium tumefaciens* (*A. tumefaciens*) C58C1 strain or similar transformed with the binary vector pMOG800 harboring the protein coding sequence. In our case they were:
 - a. pMGFP-p7B encoding GFP-fused *Melon necrotic spot virus* (MNSV) p7B
 - b. pMGFP-p7B [D₇AP₁₀A] encoding GFP-fused MNSV p7B carrying D₇AP₁₀A mutation
 - c. pMGFP-p7B [K₄₉A] encoding GFP-fused MNSV p7B carrying K₄₉A mutation
 - d. pMGFP-KDEL encoding a GFP engineered to be targeted to the ER lumen
3. Yeast extract (Difco, catalog number: 212750)
4. Tryptone (Difco, catalog number: 211705)
5. Sodium chloride (NaCl) (Panreac Applichem, catalog number: 131659)
6. Acetosyringone (Sigma-Aldrich, catalog number: D134406)
7. MES (Sigma-Aldrich, catalog number: M8250)

8. Magnesium chloride (MgCl₂) (Sigma-Aldrich, catalog number: M9272)
9. Antibiotics
 - a. Kanamycin (50 mg/ml) (Sigma-Aldrich, catalog number: K4000)
 - b. Rifampicin (50 mg/ml) (Duchefa Biochemie, catalog number: R0146)
10. Mowiol[®] 4-88 (Sigma-Aldrich, catalog number: 81381)
11. Dabco[®] 33-LV (Sigma-Aldrich, catalog number: 290734)
12. Tris base (Roche Diagnostics, catalog number: 03118142001)
13. Tris-HCl (1 M, pH 5.6)
14. Immersion oil Immersol 518 F (ZEISS, catalog number: 444960-0000-000)
15. Latrunculin B (Sigma-Aldrich, catalog number: L5288)
16. 25 μM Latrunculin B solution in 10 mM DMSO
17. LB medium (see Recipes)
18. *Agrobacterium* infiltration buffer (see Recipes)
19. Mowiol mounting medium (see Recipes)

Equipment

1. 28 °C growing chamber
2. 15 ml culture tubes
3. Swinging centrifuge rotor for 15 ml tubes
4. 1.5 ml tubes (standard Eppendorf tubes or similar)
5. BioPhotometer plus (Eppendorf)
6. 1 ml syringes without needle
7. Plant growing chamber
8. Fine paddle forceps
9. Petri culture dishes 35 x 10 mm
10. Microscope slides 76 x 26 mm (Menzel-Gläser, catalog number: AA00000112E)
11. Microscope cover slips 24 x 24 mm Nr. 1 (Menzel-Gläser, catalog number: BB024024A1)
12. Adhesive one-sided tape
13. A cork borer (one centimeter diameter) or similar cutting device such as a scalpel blade.
14. An inverted confocal microscope (ZEISS, model: LSM 780)
15. Plan-Apochromat 63x/1.40 oil objective

Software

1. Graphpad prism software (<http://www.graphpad.com>)
2. MS Excel

Procedure

A. Transient expression of proteins by agro-infiltration

1. Inoculate a single colony of transformed *A. tumefaciens* in 5 ml of LB medium with Kanamycin (50 µg/ml) and Rifampicin (50 µg/ml). Incubate the culture (12 h or more) at 28-30 °C with vigorous shaking.
2. Measure the A_{600} of the culture.
3. Collect the bacteria by slow-speed centrifugation (2,000 x g, 15 min) in a swinging rotor. Remove the supernatant.
4. Resuspend the pellet with *Agrobacterium* infiltration buffer. The final A_{600} should be adjusted to 0.2.
5. Leave at room temperature for 2 h before infiltration.
6. Infiltrate the cultures with 1 ml syringe into the lower side of the leaves. Use 3-4 week-old *N. benthamiana* plants and avoid cotyledons. Use your fingertip to apply gentle counter pressure to the other side of the leaf. A fully infiltrated part of the leaf gives a water-soaked appearance.
7. Keep the plants in growth chambers in 16 h light at 25 °C and 8 h dark at 22 °C.

B. Microscope slide preparation

1. To minimize transient overexpression artifacts in the FRAP experiments plants were used 36 h after infiltration.
2. Cut a small disk of the infiltrated leaf with a cork borer or similar device. Try to avoid the leaf veins to eliminate any potential irregularity or significant depression in the sample.
3. In the case of drug treatment, immerse tissue samples into latrunculin B (1 ml of a 25 µM solution) in a small petri culture dish (35 x 10 mm) for 1h. Perform non-treated control by tissue immersion into 10 mM DMSO.
4. Using fine paddle forceps place the leaf disk in a microscope slide with the back side facing up and mount in Mowiol mounting medium.
5. Add a coverslip and stick them to the slide with adhesive one-sided tape.
6. Place a drop of immersion oil on the cover slip over the leaf disk.

C. Fluorescence recovery after photobleaching (FRAP) assay

1. FRAP experiments were performed on an inverted Zeiss LSM 780 confocal microscope using the LSM FRAP Module from Zen 2011 software.
2. Switch on the laser lines for imaging the sample.
3. Find epidermic cells showing moderated expression levels and homogenous distribution through the ER of the GFP-tagged proteins with the Plan-Apochromat 40x/1.4 oil

objective and then switch to the Plan-Apochromat 63x/1.40 oil objective to capture images.

4. Select “range indicator”. Now image turn into one of two-color (red and blue) (Figure 1). The red areas indicated saturation of the detector due to high fluorescence intensity. Similarly, the blue area either represents no fluorescence or intensity lower than the detector can sense. Only a few pixels have to be saturated (in red), if not, adjust the pinhole, laser intensity, or detector gain.

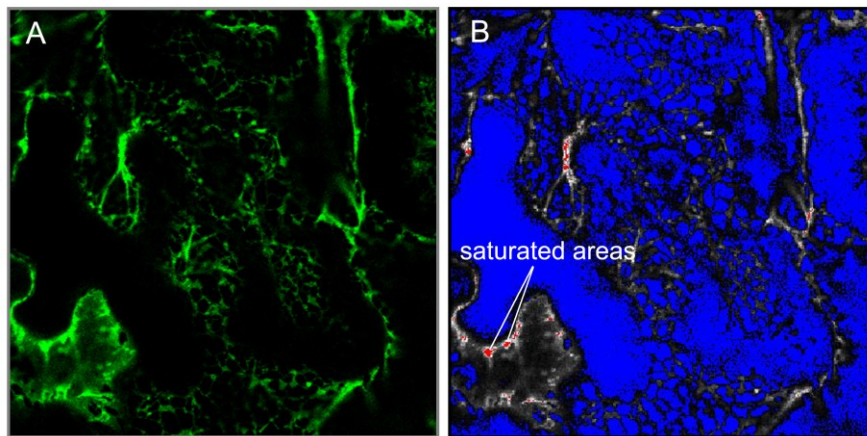


Figure 1. Laser confocal scan in green A and range indicator mode B. The saturated areas in red are indicated.

5. Use 5x optical zoom and 256 x 256 pixel resolution to take a single image of a short piece of the endoplasmic reticulum (for this purpose use “Snap” function). This zoom factor should stay the same between samples.
6. Click the bleaching option on the acquisition tab. Time series and region options will be automatically selected.
7. To define the regions of interest (ROI) which should be bleached select the circular shape in the regions menu and draw four circular ROIs of 1.7 μm diameter in fluorescent regions of the sample. Select a flat membrane region of the ER significantly larger than the bleach ROIs. Three of ROIs will be bleached and the fourth one will be used as the non-bleached reference to correct the FRAP curves for photofading caused by imaging. Locate the reference ROI as far apart as possible from the bleached ROIs. Ensure that a region which is background is in your image and draw a fifth circular ROI inside. In fast recovery rates, it is important to use small ROIs to decrease at maximum the time interval between photobleaching and imaging.

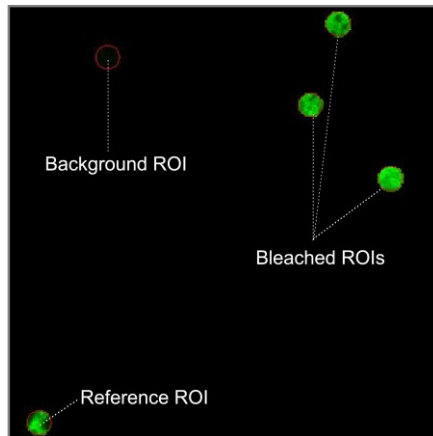


Figure 2. Spatial distribution of the region of interest (ROIs). Reference ROI must be located away from bleached ROIs to be not affected by the laser bleach. Background ROI should be in a non-fluorescent area (blue pixels in range indicator mode).

8. To setup bleaching protocol select start bleaching after 3 scans and use 100 iterations of the bleach laser over the bleach region. Do not use zoom bleach. In excitation of bleach, select the 488 nm line of the Argon laser (25% power, 100% transmission). After bleaching, try to reduce the ROI intensity between 50-75% without photodamage to the cell. After bleaching collect images at low laser power (25% power, 2% transmission).
9. Open the time series menu and specify the total number of images in the final data set (before and after bleaching) by setting the number of cycles to 100. This results in an interval between the beginning of each scan of approximately 191 msec. To further increase the rate of data acquisition, use bidirectional scanning.
10. Start FRAP experiment by pressing “Start Experiment button” and examine the FRAP curve on the computer. ROI reference intensity should remain constant or be slightly reduced due to photofading during image acquisition Repeat FRAP on different cells and days to ensure reproducibility. To allow comparison between different cells and proteins, the detector gain can be readjusted but all other settings must be kept the same.

D. FRAP data processing

1. FRAP data generated using the LSM systems can be displayed and analyzed with the confocal operating software. Alternatively, raw fluorescence intensity values from bleached, background and reference ROIs can be exported to Microsoft Excel to be double normalized (Phair *et al.*, 2004).
2. Calculate the photobleaching rate (R) by comparing the intensity of the fluorescence of the reference ROI before (RF_0 , average intensity of the three scans taken before bleaching) and at each scan after photobleaching (RF_n) $R_n = F_n/F_0$.

3. Normalize the fluorescence intensity of the bleached ROIs (Bleached F_n) as follows: $NF = (BleachedF_n - BackgroundF_n)/r$.
4. Copy the data to a program such as GraphPad Prism and curve fit the fluorescence by using non-linear regression and the exponential one-phase association model. The mobile fraction (Mf) that corresponds to the plateau value and half-life $\tau_{1/2}$, the time it takes for fluorescence intensity to reach half the maximum of the plateau level are obtained by the software.

Notes

1. Every FRAP protocol need to be adapted depending on the nature of sample and preparation. FRAP setup shown herein is merely orientative and should not be considered as guarantee of good results. Then, it is very likely your first session efforts will be focused in optimizing photobleaching and data acquisition (Carisey *et al.*, 2011).

Recipes

1. LB medium
 Mix 5 g of yeast extract with 10 g of tryptone, 10 g of NaCl
 Add dH₂O to 1,000 ml
2. *Agrobacterium* infiltration buffer
 10 mM MES (pH 5.6)
 10 mM MgCl₂
 150 μ M acetosyringone
3. Mowiol mounting medium
 - a. Add 9.6 ml of Mowiol 4-88 to 24 ml of glycerol and 24 ml of dH₂O.
 - b. Stir to mix and leave for several hours at room temperature.
 - c. Add 9.6 ml of 1 M Tris-Cl (pH 8.0) and 38.4 ml of dH₂O heat to 50 °C for 10 min with occasional mixing.
 - d. After the Mowiol dissolves (approximately 3 h), clarify by centrifugation at 5,000 x g for 15 min.
 - e. For fluorescence detection, add Dabco to 2.5% to reduce fading.
 - f. Aliquot in 1.5 ml tubes and stored at -20 °C.

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