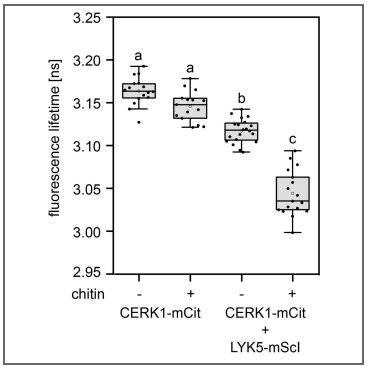

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Protocol status: Working
We use this protocol and it's working

FLIM-FRET analyses with mCitrine / mScarlet-I -tagged chitin receptors stably expressed in A. thaliana

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ABSTRACT

Elucidating protein-protein interactions is crucial for our understanding of molecular processes within living organisms. Microscopy-based techniques can detect protein-protein interactions *in vivo* at the single cell level and provide information on their subcellular location.

Fluorescence Lifetime Imaging Microscopy (FLIM) - Förster resonance energy transfer (FRET) is one of the most robust imaging approaches, but it is still very challenging to apply this method to proteins which are expressed under native conditions. We have established a novel combination of fluorescence proteins (FPs), mCitrine and mScarlet-I, which is ideally suited for FLIM-FRET studies of low abundance proteins expressed from their native promoters in stably transformed plants. The donor mCitrine displays excellent brightness in planta, near-monoexponential fluorescence decay and a comparatively long fluorescence lifetime.

In this protocol, we describe FLIM-FRET experiments for CERK1-mCitrine and LYK5-mScarlet-I or CERK1-Scarlet-I in stably transformed Arabidopsis lines.

MATERIALS

Primary antibodies for Western blots:
for mCitrine: rat monoclonal anti-GFP (Chromotek, 3H9),
for mScarlet-I: rat monoclonal anti-RFP (Chromotek, 5F8)

Secondary antibodies for Western blots:
Goat-Anti-Rat AP-conjugate (Sigma-Aldrich, A8438)
Goat-Anti-Rabbit AP-conjugate (Sigma-Aldrich, A3687)

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Keywords: Förster resonance energy transfer (FRET), fluorescence lifetime imaging (FLIM), stably transformed plants, in planta FLIM-FRET, mCitrine, mScarlet-I, Co-Immunoprecipitation, Arabidopsis

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If signals are weak:
use Pierce Western Blot Signal Enhancer (Thermo Fisher Scientific, 21050)

Detection substrate:
Immunstar-AP (Bio-Rad, 1705018)

Detection system:
Bio-Rad Chemidoc

High purity chitin (Sigma Aldrich /Merck, C9752).

Rough-surfaced glass pistils that fit 1.5ml microcentrifuge tubes (Glasgeraetebau Ochs, Bovenden, Germany).

IKA RW 20 overhead stirrer

Microscopy slides
Cover slips

Leica Microsystems TCS SP8 Falcon system equipped with HyD SMD detectors, a pulsed white light laser and a HC PL APO 40x/1.10 W CORR CS2 objective or similar.

Generation of transgenic lines expressing mCitrine- and mSca...

1 Clone the two proteins of interest you wish to test for interaction in frame with mCitrine and mScarlet-I. A tested vector for this purpose is pGreenII-0229 (Hellens *et al.*, 2000). Transform constructs into *Arabidopsis thaliana* (for example according to Clough and Bent, 1998). Select transformants and confirm expression and correct localisation of fusion proteins by confocal microscopy (see section "imaging" below). Check accumulation of the full-length fusion proteins by Western Blotting. Tested primary and secondary antibodies as well as other Western blot reagents are listed in the materials section.
Generate double transgenic lines by crossing or double transformation. Check again for accumulation and integrity of the fusion protein by microscopy and Western blotting.

2 The protocol outlined below has been established for the chitin receptor component pairs

pCERK1-CERK1-mCitrine + pLYK5-LYK5-mScarlet-I
and
pCERK1-CERK1-mCitrine + pCERK1-CERK1-mScarlet-I.

Their interaction can be triggered by the ligand chitin.

Chitin preparation

3 Since chitin is insoluble in water, a fine suspension has to be prepared to avoid sedimentation and ensure efficient, uniform treatment of plant tissues:

4 Weigh out 10mg of high purity chitin (see materials) into a 1,5 ml microcentrifuge tube.

5 Add 200µl water and grind to a smooth paste with a overhead stirrer equipped with a glass pistil (see materials).

6 Rinse the pistil twice with 200µl water and finally add 400µl water to make 1 ml of a 10 mg/ml stock. The suspension should appear milky white with minimal sedimentation. It can be kept in the fridge for about 1 week, but should not be frozen.

Chitin infiltration

7 Grow transgenic lines under short day conditions (8h light at 22°C / 16h dark at 18°C, relative humidity 65%) for approximately 4 weeks.

8 From the rosette, cut out leaf pieces using a scalpel or biopsy punch (maximum size approximately 1 cm x 1 cm). If you want to compare chitin-treated to water control samples, cut out two leaf pieces from the left and right side of the same leaf.

9 From two 5 ml Luer-Lock syringes, remove the plungers and close the tips with Luer-Lock syringe caps (also called combi-stoppers).

10 Pipette 1 ml water into one of the syringes and 1 ml 100 µg/ml chitin suspension into the other. Place the leaf piece(s) inside the syringes using forceps.

11 For each syringe, replace the plunger and hold the syringe so that the tip points upwards.

12 Take the cap off and remove any air by pushing the plunger in.

13 Put the cap back on.

14 Apply a partial vacuum by sharply pulling the plunger back several times.
The leaf pieces should now appear translucent.

Imaging

15 Mount the leaf piece(s) on a microscopy slide in approximately 50µl water (abaxial side facing up) and put on a cover slip.

16 On a Leica Microsystems TCS SP8 Falcon system, activate two HyD SMD detectors and the pulsed white light laser. Choose a Leica HC PL APO 40x/1.10 W CORR CS2 objective or similar.

17 For the mCitrine channel, set excitation to 514 nm and the detection window between 525-560 nm (detecting mCitrine). Set a second detection window at 585-660 nm (control for mScarlet-I bleed through).

18 For the mScarlet-I channel, set excitation to 570 nm and the detection window between 585-660 nm (detecting mScarlet-I). Set a second detection window at 525-560 nm (control for mCitrine bleed through).

19 Set laser repetition rate to 80 mHz and adjust intensity in order to get good signals, but avoid saturated areas. Set scanner speed to 200-400 Hz. If the signal is very weak, use accumulation mode (accumulate 2-4 frames).

20 To exclude chloroplast autofluorescence signals, set a fluorescence lifetime gate between 0.4-6 ns.

21 Acquire images in sequential scanning mode. This is necessary obtain mScarlet-I signals without mCitrine bleed through.

Fluorescence Lifetime Imaging and Calculations

22 If not already done for regular imaging, activate two HyD SMD detectors and the pulsed white light laser on the Leica Microsystems TCS SP8 Falcon system. Choose a Leica HC PL APO 40x/1.10 W CORR CS2 objective or similar.

23 For measuring mCitrine donor lifetime, set excitation to 514 nm and the detection window between 525-560 nm (detecting mCitrine). Set a second detection window at 585-660 nm (mScarlet-I and autofluorescence control).
Setting up the laser and detection windows specifically for mScarlet-I is not necessary for most types of experiment.

If you do wish to measure mScarlet-I fluorescence intensity and lifetime (e.g. in an acceptor only control), use the same settings as described for regular imaging and acquire images in sequential scanning mode. If acceptor fluorescence intensity and lifetime are not needed, excite only with the 514 nm laser to avoid unnecessary bleaching.

24 Start the FLIM tab of the LASX 3.5.5 (or similar) software. Set the number of acquired frames to a suitable number (e.g. 20). If the specific signal is much stronger than any autofluorescence, an intensity threshold for acquisition may be set.

25 Set laser repetition rate to 40 mHz and scanner speed to 400 Hz. Adjust laser intensity so that the maximum photon count rate per pixel does not exceed 1 photon per laser pulse (no or only very few events above the red line in the pixel intensity histogram).

26 Record at least 10 images for each fluorescence protein and/or condition.

27 For each image, select the region of interest (ROI) using a suitable tool (e.g. polygon tool for PM and cell periphery, ellipse tool for nuclei). In this step it is important to avoid regions with autofluorescence (plastids, stomata, cuticle), or areas with air inclusions. Inspect fast-FLIM renderings of images recorded between 525-560 nm and 585-660 nm to identify such structures.

28 Select a fit model that is suitable for your experiment (n-exponential reconvolution or n-exponential tail fit with one or more exponential components). Inspect the fit curve, residuals and χ^2 values to aid the decision. If the fit does not match the recorded histogram well and/or χ^2 is high (>5) increase the number of exponential components. For mCitrine without any FRET, a mono-exponential reconvolution model is usually appropriate. When FRET occurs, a bi-exponential model is required to fit the data. Therefore we typically calculate fluorescence lifetimes with both models.

29 Apply the selected model to the ROIs of all images. To do so, highlight them in the FLIM-Table and choose "fit all".

30 Export FLIM-Table to visualize fluorescence lifetimes as box plots and apply statistical analyses (using GraphPad Prism, Origin or other software).

31 For generation of fluorescence lifetime images, apply a precise FLIM image fit to selected pictures. Set the threshold for counts per pixel to ≥ 50. If a considerable proportion of pixels with specific signal is below this number, apply pixel binning.

32 For FRET-Calculations select fit model (mono- or multi-exponential donor). The mono-exponential donor model is suitable for mCitrine recordings with good fluorescence intensity. If the signal is very weak (weaker than chloroplast autofluorescence), autofluorescence will contaminate the specific fluorescence signal despite careful ROI selection. In this case choose the multi-exponential donor model. For mCitrine, set the Förster Radius to 6.333 nm and provide the unquenched donor lifetime (average lifetime of at least 10 donor-only measurements).

33 Fit the FRET model to all ROIs and export the FRET-Table vor visualisation and statistical analysis.