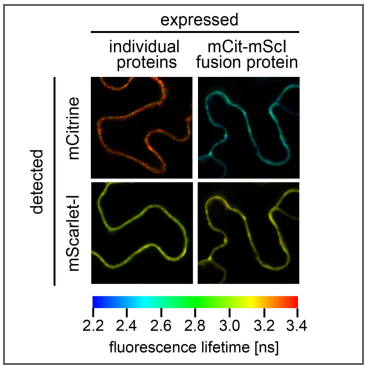

OCT 29, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.6qpvr38epvmk/v1

Protocol Citation: Elena-Kristin.Petutschnig, leon.pierdzig, Josephine Mittendorf, Jule Meret Niebisch, Volker Lipka 2023. FLIM-FRET analyses with mCitrine-mScarlet-I transiently expressed in N. benthamiana . **protocols.io** https://dx.doi.org/10.17504/protocols.io.6qpvr38epvmk/v1

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

FLIM-FRET analyses with mCitrine-mScarlet-I transiently expressed in N. benthamiana

Elena-Kristin.Petutschnig[1], leon.pierdzig[1], Josephine Mittendorf[1], Volker
Jule Meret Niebisch[1], Lipka[1]

[1]Department of Plant Cell Biology, Albrecht-von-Haller-Institute for Plant Sciences, Georg-August-University

Elena-Kristin.Petutschnig

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 control proteins in *Nicotiana benthamiana*, namely mCitrine (donor only control), mScarlet-I (acceptor only control) and a mCitrine-mScarlet-I fusion protein (FRET control).

MATERIALS

N. benthamiana plants (5 weeks old, grown under long day conditions [16h/8h] at 25°C/22°C and 65% humidity)

Agrobacterium tumefaciens GV3101 harboring pSoup (Hellens *et al.*, 2000)

mCitrine and mScarlet-I constructs (Petutschnig *et al.*):
pGreenII-0229-p35S-mCitrine

Created: Aug 27, 2023

Last Modified: Oct 29, 2023

PROTOCOL integer ID: 87010

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

Funders Acknowledgement:
ERA-CAPS SIPIS
Grant ID: LI1317/8-1

pGreenII-0229-p35S-mScarlet-I
pGreenII-0229-p35S-mCitrine-mScarlet-I

LB media:
10g/l peptone (Gibco 211677)
5g/l yeast extract (Gibco 212750)
10g/l $NaCl_2$ (Carl Roth 3957)
for plates 15g/l agar (BD Difco, 214530)

Antibiotics:
Rifampicin, (Duchefa Biochemie R0146), final concentration 20µg/ml
Gentamycin, (Duchefa Biochemie G0124), final concentration 50µg/ml
Kanamycin, (Carl Roth T832), final concentration 50µg/ml
Tetracycline, (Carl Roth 0237), final concentration 5µg/ml

Infiltration medium:
10mM $MgCl_2$ (Carl Roth 2189)
10mM MES pH5,4 (Carl Roth 4256)
150µM acetosyringone (SigmaAldrich D134406)

5 ml Luer-Lock syringe (MediWare I3 0502)

Luer-Lock Combi-Stopper syringe caps (B.Braun 4495101)

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.

Agrobacterium transformation

1 Transform suitable *Agrobacterium tumefaciens* strain and select transformants on LB plates containing appropriate antibiotics.
For the control constructs pGreenII-0229-p35S-mCitrine, pGreenII-0229-p35S-mScarlet-I and pGreenII-0229-p35S-mCitrine-mScarlet-I transform *Agrobacterium tumefaciens* GV3101 harboring pSoup (Hellens *et al.*, 2000) and select on LB plates containing 20µg/ml rifampicin, 50µg/ml gentamycin, 50µg/ml kanamycin and 5µg/ml tetracycline.

Transient expression in *Nicotiana benthamiana*

4d 2h

2 Inoculate *Agrobacteria* cultures and grow to an OD_{600} of 0.8-1.2 at 28°C. 2d

3 Pellet the culture and resuspend in infiltration medium (see materials) at an OD_{600} of 0.2.

4 Incubate at room temperature for 2 h. 2h

5 Infiltrate suspension into leaves from the abaxial side with a needleless syringe. Infiltrate pGreenII-0229-p35S-mCitrine (donor only control), pGreenII-0229-p35S-mScarlet-I (acceptor only control) and pGreenII-0229-p35S-mCitrine-mScarlet-I (FRET control) individually. Also infiltrate a 1:1 mixture of the pGreenII-0229-p35S-mCitrine and pGreenII-0229-p35S-mScarlet-I suspensions to generate a non-interacting control.

6 Incubate plants in a growth chamber or cabinet at long day conditions (16h light, 25°C / 8h dark, 22°C) for 2-3 days. 2d

Removing air from leaf tissues

7 Cut out transformed leaf pieces using a scalpel or biopsy punch (maximum size approximately 1 cm x 1 cm).

8 From a 5 ml Luer-Lock syringe, remove the plunger and close the tip with a Luer-Lock syringe cap (also called a combi-stopper).

9 Pipette 1 ml water into the syringe and place the leaf piece(s) inside it using forceps.

10 Replace the plunger and hold the syringe so that the tip points upwards.

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

12 Put the cap back on.

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

Imaging

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

15 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.

16 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).

17 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).

18 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 signal is very weak, use accumulation mode (accumulate 2-4 frames).

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

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

Fluorescence Lifetime Imaging and Calculations

21 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.

22 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, 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.

23 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.

24 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).

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

26 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.

27 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.

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

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

30 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.

31 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).

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