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Immunoprecipitation and FRET-FLIM to determine metabolons on the plant ER

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Abstract:

Metabolons are protein complexes that contain all the enzymes necessary for a metabolic pathway but also scaffolding proteins. Such a structure allows efficient channeling of intermediate metabolites form one active site to the next and is highly advantageous for labile or toxic intermediates. Here we describe two methods currently used to identify metabolons via protein-protein interaction methodology: immunoprecipitations using GFP-Trap®_A beads to find novel interaction partners and potential metabolon components and FRET-FLIM to test for and quantify protein-protein interactions *in planta*.

Key words: endoplasmic reticulum, metabolon, enzymes, protein complex, protein-protein interaction, co-immunoprecipitation, GFP-trap, FRET-FLIM

1. Introduction

The plant ER has been shown to be dynamic and constantly remodelling [1]. Specific

ER-microdomains with unique lipid composition are suggested to direct proper anchoring of proteins and to facilitate and stabilize the subsequent assembly of components required to form a specific protein complex capable of carrying out a complete metabolic pathway [2]. These so called metabolons enable metabolic processes to take place in the necessary highly coordinated and regulated processes in an efficient manner. Metabolons contain sequential enzymes plus scaffolding proteins allowing for efficient channeling of metabolic intermediates from one active site directly to the next [2]. The concept of these so called metabolons was introduced in 1985 by Paul Srere in the context of glycolytic and Krebs cycle enzymes [3]. Sequential enzymes of a specialised biosynthetic pathway are transiently linked together by noncovalent binding typically stabilised by membrane or cytoskeletal anchoring [4]. Such a mechanism increases substrate concentration and turnover rates, prevents unwanted diffusion and metabolic interference, and is beneficial for containment of labile or toxic intermediates. Such complex organization has been shown for protein translocases in the endoplasmic reticulum, in chloroplasts and mitochondria [5, 6, 7] allowing coordinated and efficient transport potentially also preventing back-flux.

Metabolons will typically be comprised of sequential enzymes in the pathway together with scaffolding proteins allowing for efficient channeling of metabolic intermediates from one active site to the next [2]. Candidate proteins for such scaffolding are chaperones as well as membrane-anchored cytochrome P450 enzymes that can serve as nucleation points and platforms for metabolon formation [2]. In addition to this TA proteins were shown to have the potential to localise metabolons to specific organelle surfaces; e.g. in the TOM complex where the tail-anchors operate as assembly signal [8]. This can also stabilise proteins

compared to their cytosolic versions as shown for tobacco expression of the HIV gene product Nef which has been researched in the context of HIV vaccination [9].

Also membrane-structural proteins have been suggested to play a role in metabolon formation, e.g. P450 enzymes involved in a lignin biosynthetic metabolon were co-purified with reticulon proteins (RTN) [10] which contribute to ER tubule shaping [11].

In plants, metabolons have been shown for several enzymatic pathways in secondary and primary metabolism [12]. Most metabolons involved in secondary metabolism involve less stable interactions than described for metabolons in primary metabolism demanding refined microscopy methods and computational analysis to prove their interactions [13].

Metabolons have been shown e.g. for the production of flavonoids [14] and sporopollenin [15] in arabidopsis and the glucoside dhurrin [16] in sorghum. Recently a soybean isoflavonoid metabolon tethered to the ER has been reported [17]. An ER-localised metabolon for the biosynthesis of the auxin indole-3-acetic acid (IAA) has been suggested [18].

The metabolon complexes producing phenylpropanoids [19] and flavonoids [14, 20] seem to cater for specific metabolites made from shared intermediate products. The enzyme composition in the flavonoid pathway complex can change resulting in varying end products [12, 14]. Membrane-anchored cytochrome P450 enzymes are reported to serve as nucleation points and platforms for the metabolon formation. Most P450 monooxygenases are membrane-bound to the ER via an N-terminal tether enhancing metabolon formation on this membrane system [2, 21].

Metabolons for the glucoside dhurrin in sorghum are formed in specific in ER domains [22] and

metabolon formation between the three enzymes involved in dhurrin biosynthesis is suggested to be highly advantageous as the dhurrin precursor p-hydroxymandelonitrile is very unstable.Another ER-localised metabolon the biosynthesis of sporopollenin in tapetal cells. Colocalisation and protein-protein interaction of these enzymes indicate the existence of a sporopollenin metabolon on the ER [15]. This benefits a coordinated and quick production of pollen cell wall components allowing for rapid pollen development.

Molecular distances (1-10 nm) can be measured using energy transfer processes. Förster or fluorescence resonance energy transfer (FRET), first described by Theodor Förster over 50 years ago [23] relies on the non-radiative energy transfer from an excited fluorescent donor molecule to a different non-excited fluorescent acceptor molecule in its vicinity. The very short distances required for this process to occur (<10 nm) means the two molecules, in this case two proteins, needs to be physically close to one another. For FRET to occur the donor emission spectrum must overlap sufficiently with the acceptor absorption spectrum, the donor and acceptor dipoles display a mutual molecular orientation. The rate of the energy transfer k_T is described and calculated using Equation 1:

$$k_T = \left(\frac{1}{\tau_D}\right)\left(\frac{R_0}{R}\right)^6 \qquad (1)$$

where τ_D is the donor excited-state lifetime in the absence of the acceptor, *R* is the distance between *D* and *A,* and R_0 is the Förster radius. At the Förster radius, 50% of the donor molecules will emit fluorescence while the rest will undergo energy transfer. Since the energy transfer process is strongly distance dependent with $1/R^6$, FRET can be used to measure distance and examine molecular interactions on a nm spatial scale. During FRET, the rate of decay is reduced by a quenching process that depletes the excited state of the donor

fluorophore, i.e. the donor fluorescence lifetime is shortened. By measuring changes in the excited state lifetime of the donor at each pixel making up an image, steady state FRET is enhanced. This is described as fluorescence lifetime imaging microscopy or FRET-FLIM. Generally the two proteins under investigation are tagged with GFP and its variants. We have used GFP and RFP as the donor and acceptor respectively in our work. We describe here two methods that have been used in metabolon work: Immunoprecipitations using GFP-Trap®_A beads to find novel interaction partners and potential metabolon components and FRET-FLIM to test for and quantify protein-protein interactions *in planta*

2. Materials

2.1 Buffers and Media for immunoprecipitations

1 Murashige and Skoog (MS) medium: 4.4 g/l Murashige and Skoog basal salts, pH5.7, 10 g/l agar.

2 Lysis buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40, 1mM PMSF, protease inhibitor according to manufacturers' instructions.

3 Equilibration buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA.

2.2 Buffers and Media for FRET-FLIM

For plant infiltration and expression in tobacco see protocols in chapter "Labelling the ER for light and fluorescence microscopy"

2.3 Equipment for immunoprecipitations

1 Petri dishes (large, with lid L x W x H: 245 mm x 245 mm x 25 mm)

2 50 ml Falcon tubes

3 Glass bottles for buffers

4 Cheese cloth

5 Refrigerated table centrifuge

2.4 Equipment for FRET-FLIM

1 At least two-channel confocal and one-channel FLIM set up

2 Becker and Hickl SPC 830 or SPC150 time correlated single photon counting card

3 SPCImage analysis software version 5.1 (Becker and Hickl)

3. Methods

3.1 Immunoprecipitation using GFP-Trap®_A beads

(This part of the procedure will take between 4 and 5 h depending on sample size.) This protocol uses GFP-Trap®_A beads (Chromotek, Martinsried, Germany) and the procedure is carried according to the company's protocol with slight modifications (*see* **Note 1**): *Arabidopsis thaliana* seedlings expressing the protein of interest fused to a fluorescent tag (see Protocol "Labelling the ER for light and fluorescence microscopy" for preparation of stable arabidopsis transformants) are grown for 2 weeks on MS plates (*see* **Note 2**).

1. Approximately 5 g of whole seedling plant material are ground first in liquid nitrogen to a fine powder and then in 5 ml lysis buffer until liquid. The extract is then distributed into three 2-ml-Eppendorf tubes.
2. The extracts are incubated on ice for 30 min and then centrifuged at 10,000g for 10 min at 4°C.
3. The supernatant (about 4 ml) is poured into fresh 50 ml Falcon tubes via 2 layers of cheese cloth.

4. 100ul of GFP-Trap®_A beads per sample are equilibrated in 500 µl equilibration buffer and centrifuged at 2,500 g for 2 min. The supernatant is being discarded and this wash is repeated twice.
5. 100 µl of the washed beads are added to the plant extract and the mixture is shaken on ice for 2 hours with the tubes being placed horizontally in the ice.
6. After this, tubes are centrifuged at 2,500 g for 2 min at 4°C and the supernatant is being carefully discarded without disturbing the agarose pellet.
7. The resulting agarose pellet is then washed twice with dilution buffer.
8. This pellet can be use straight away for tryptic peptide digest and mass spectrometry analysis for containing proteins [24].

3.2 Real time imaging of metabolon protein-protein interactions using two photon FRET-FLIM

Potential interaction candidates resulting from the immunoprecipitation are tested for interactions in planta with the bait protein. This procedure is done using agrobacterium mediated transient expression in tobacco leaf epidermal cells (see Protocol “Labelling the ER for light and fluorescence microscopy” for transient expression in tobacco).

1. Epidermal samples of infiltrated tobacco leaves are excised, and both confocal/multiphoton FRET-FLIM data capture is performed by a two photon microscope [25] (*see* **Note 3**).
2. The two-photon microscope is built around a Nikon TE2000-U inverted microscope is used with a modified Nikon EC2 confocal scanning system to allow for near infra-red laser wavelength for FLIM [26].
3. Laser light at a wavelength of 920 nm is produced by a mode-locked titanium sapphire laser (Mira; Coherent Lasers), with 200-fs pulses at 76 MHz, pumped by a solid-state continuous wave 532-nm laser (Verdi V18; Coherent Laser).

4. The laser beam is focused to a diffraction limited spot using a water-immersion objective (Nikon VC; 360, numerical aperture of 1.2) to illuminate specimens on the microscope stage.
5. Fluorescence emission is collected without descanning (no pin-hole), bypassing the scanning system, and passed through a BG39 (Comar) filter to block the near-infrared laser light. Line, frame, and pixel clock signals are generated and synchronized with an external detector in the form of a fast microchannel plate photomultiplier tube (Hamamatsu R3809U).
6. Linking these via a time-correlated single-photon counting PC module SPC830 (Becker and Hickl) generated the raw FLIM data. Prior to FLIM data collection, the GFP and mRFP expression levels in the plant samples within the region of interest are confirmed using a Nikon EC2 confocal microscope with excitation at 488 and 543 nm, respectively.
7. A 633-nm interference filter is used to significantly minimize the contaminating effect of chlorophyll autofluorescence emission that would otherwise obscure the mRFP as well as GFP emission.
8. Data are analysed by obtaining excited-state lifetime values first on a pixel by pixel basis then of a region of interest on the nucleus, and calculations are made using SPCImage analysis software version 5.1.
9. The distribution of lifetime values within the region of interest is generated and displayed as a curve. Only values with a χ^2 between 0.9 and 1.4 are taken.
10. The median lifetime, minimum and maximum values for one-quarter of the median lifetime values from the curve are taken to generate the range of lifetimes per sample.
11. The ER associated with at least three nuclei from a minimum of three independent biological samples per protein-protein combination are analysed, and the average of the ranges is taken.
12. The degree or efficiency of energy transfer (E), from one protein to the other may be determined using equation 2:

$$E\% = \left[1 - \left(\frac{\tau_{DA}}{\tau_D}\right)\right] \times 100 \qquad (2)$$

Where τ_D and τ_{DA} are the measured excited state lifetime of the donor and acceptor respectively.

Example data is shown in Figure 1.

4. Notes

1. Gloves have to be worn for the whole procedure to reduce contamination.
2. This protocol can also be carried out with tobacco transiently expressing the protein of interest but will result in reduced yield of interacting proteins and will of course only show interactors in tobacco.
3. Best measurement results for the ER have been shown for the nuclear envelope and associated ER. Latrunculin B can be applied to the leaf discs to depolymerize the actin cytoskeleton and therefore inhibit any movement of the ER. This may not be necessary with high expression levels of the donor protein which result in shorter time for data capture and therefore movement is less critical.

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Figure legends

Figure 1: FRET-FLIM analysis of TAR2 without an interaction partner (A–E) or with YUC8 (F–J). (A, F) Raw FRET-FLIM data; (B, G) pseudocoloured lifetime maps showing the lifetime values for each point within the region of interest; (C, H) distribution of lifetimes across the image. Blue shades represent longer GFP fluorescence lifetimes than green ones. (D, I) Representative decay curves of a single point with an optimal single exponential fit, where $\chi 2$ values from 0.9 to 1.2 were considered an excellent fit to the data points (a binning factor of 2 was applied). The confocal images for the analysis in (E, J) show the GFP construct in green and the mCherry construct in red. This example of FRET-FLIM analysis shows TAR2–GFP alone as a negative control and YUC8 for protein–protein interaction. The fluorescence lifetime values for TAR2–GFP+YUC8–mCherry are 2.8 ± 0.02 ns and therefore statistically lower than the lifetime values for the TAR2–GFP fusion alone (3.04 ± 0.03 ns).