FRET-FLIM as an approach to validate proteomics datasets

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Introduction

The cortical endoplasmic reticulum (ER) in plants is pleomorphic and structured as a tubular network capable of morphing into flat cisternae, mainly at three way junctions, and back to tubules. Plant reticulon (RTNLB) proteins tubulate the ER by dimer- and oligomerization, creating localised ER membrane tensions that result in membrane curvature. Some RTNLB ER-shaping proteins are present in the plasmodesmal (PD) proteome (Fernandez-Calvino et al., 2011) and may contribute to the formation of the desmotubule, the axial ER-derived structure that traverses primary PD (Knox et al., 2015). Here we investigate the binding partners of two PD-resident reticulon proteins, RTNLB3 and RTNLB6, which are located in primary PD at cytokinesis (Knox et al., 2015).

We used a dual approach to identify interacting partners of RTNLB3 and RTNLB6 (Fernandez-Calvino et al., 2011; Knox et al., 2015). First, GFP-immunoprecipitation assays coupled to mass spectrometry to identify proteins potentially binding to RTNLB3 and RTNLB6 was performed. Second, a detailed FRET-FLIM (Förster Resonance Energy Transfer by Fluorescence Life Time Imaging) analysis was conducted with the identified proteins to confirm prey-bait interactions in vivo.

Our mass spectrometry interaction data identified a large percentage (40%) of ER proteins, including other RTNLB family members. However, we also found a relatively large number (25%) of proteins present in the published PD proteome (Fernadez-Calvino et al., 2011), and a surprisingly high proportion (35%) of plasma membrane proteins. Of the PD-resident proteins we identified, a significant number were shown previously to be targets of viral movement proteins (MPs). Additional proteins identified suggested roles for RTNLBs in transport and pathogen defence. We suggest that RTNLBs may play key roles in anchoring and/or signalling between the cortical ER and PM. This proteomics data was subsequently validated through in vivo assessment of interactions by FRET-FLIM analysis.

FRET-FLIM assays on the plant ER

The application of time resolved fluorescence spectroscopy to imaging biological systems enabled the design and implementation of Fluorescence Lifetime Imaging Microscopy (FLIM). The technique allows the measurement and determination of the space map of picosecond fluorescence decay at each pixel in the image through confocal single and multiphoton excitation. The general fluorescence or Förster resonance energy transfer (FRET) to determine co-localisation of two colour chromophores can be improved to determine physical interactions using FRET-FLIM using protein pairs tagged with appropriate GFP-fluorophores and monomeric red fluorescent protein. FRET-FLIM measures the reduction in the excited state life time of GFP (donor) fluorescence in the presence of an acceptor fluorophore (e.g. mRFP). The observation of such a reduction is an indication that the two proteins are within a distance of 1 to 10 nm thus indicating a direct physical interaction between the two protein fusions (Osterrieder et al., 2009, Sparkes et al., 2010). It was previously shown that a reduction of as little as ~200 ps in the excited state lifetime of the GFP labelled protein represents quenching through a protein-protein interaction (Stubbs et al., 2005). In this study, due to limitations in the speed of photon counting of the FLIM apparatus, measurements were taken from high expressing areas of ER regions with relatively low mobility, such as the ER associated with the nuclear envelope. This allowed more reliable measurements than obtainable from the fast moving cortical ER. Furthermore, to allow consistent and reliable measurements, proteins that usually localise to PD or plasma membrane (PM) were driven to the ER by overexpression.

RTNLB3-GFP alone showed a fluorescence lifetime of 2.47±0.05 ns, and RTNLB6-GFP alone a lifetime of 2.63±0.06 ns. Figure 1 shows the FRET-FLIM analysis steps for RTNLB6-GFP alone (A-D) as a negative control, and for RTNLB6-GFP interacting with RFP-RTNLB6 (E-I) as a positive control. Raw FLIM images are shown in A and E. This analysis takes into account the lifetime values of each pixel within the image visualised by a pseudo-coloured lifetime map (B and F). The graph shows the distribution of lifetimes within the image (C and G) with blue shades representing longer GFP fluorescence lifetimes than green ones. Decay curves (D and H) of a representative single pixel highlight an optimal single exponential fit, where Chi square ($\chi 2$) values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2). Confocal pictures for the region of interest showing the GFP-construct in green and the mRFP-construct in red are shown in A (inset) and I. This specific example shows that RTNLB6 homodimerizes because the lifetime values for the GFP/mRFP fusion pair (2.38±0.01 ns, Table 3) are significantly lower than those for the GFP fusion alone (2.63±0.06 ns).

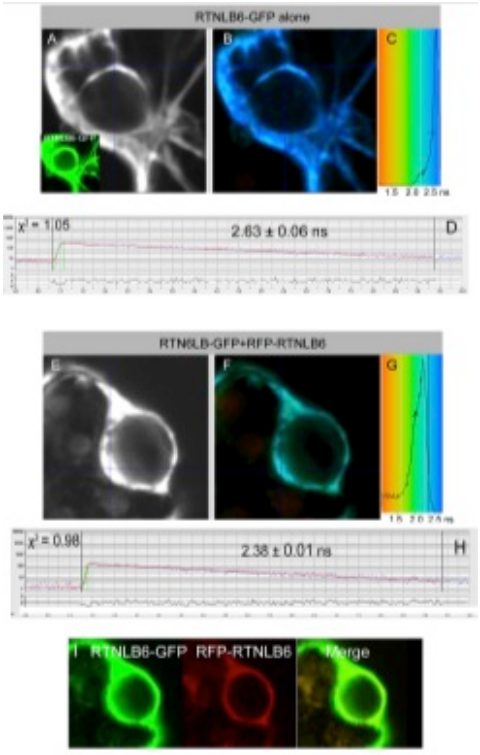

Figure 1: FRET-FLIM analysis of RTNLB6 without an interaction partner (A-D) or RTNLB6 dimerization (E-I). Images A and E display the raw FRET-FLIM data. In B and F pseudo-coloured lifetime maps show the lifetime values for each point within the region of interest while the distribution of lifetimes across the entire image is shown in C and G with blue shades representing longer GFP-fluorescence lifetimes than green ones. Images D and H display representative decay curves of a single point with an optimal single exponential fit, where Chi square ($\chi 2$) values from 0.9 to 1.2 were considered an excellent fit to the data points (binning factor of 2. Images A (inset) and I are the respective confocal images for the analysis showing the GFP-construct in green and the mRFP-construct in red. This example of FRET-FLIM analysis shows that RTNLB6 homodimerizes because the lifetime values for the GFP/mRFP fusion pair (image H, 2.38±0.01 ns) are lower than those for the GFP fusion alone (image D, 2.63±0.06 ns). White bar = 5µm.

FRET-FLIM analysis to validate immunoprecipitation data

17 proteins from the list of 146 potential interacting proteins were subjected to further analysis to test for interactions in vivo using FRET-FLIM(Table 1). The choice of these 17 proteins was based on four main criteria:

i) Known or expected interacting partners of RTNLBs from our published work as positive controls; RTNLB3 and RTNLB6 (RTNLB3 in Sparkes et al., 2010) and RHD3/RL2 (Lee et al., 2013).

ii) Proteins present in the PD proteome.

iii) A selection of high- and low-abundance proteins distributed throughout the quantitative lists. These were used to test the hypothesis that proteins with low abundance in the GFP-immunoprecipitation assays were likely to represent weak or false-positive interactions. For example, thioredoxin 3 (TRX3) showed very low abundance relative to, for example, DWARF1.

iv) TCP-1/cpn60 chaperonin family protein (TCP1) was chosen as a further control as this protein was found in the proteome for RTNLB3 but not for RTNLB6.

3 out of the 17 proteins identified by mass spectrometry did not show interaction in the FRET-FLIM approach. Significantly, TCP1 that was pulled down with RTNLB3 but not with RTNLB6 showed indeed only interaction with RTNLB3 in the FRET-FLIM but not with RTNLB6 validating both the proteomics data and the FRET-FLIM approach as a confirmatory method.

		% in total spectra			FRET-FLIM			present in 2nd MS run	
Proteins	Accession No.	RTNLB3-1	RTNLB6 -1		RTNLB3-1	RTNLB6 -1		RTNLB3-2	RTNLB6 -2
Reticulan like protein RTNLB3 *	AT1G64090.1	0.042%	0.036%		+	+		+	+
Reticulon family protein RTNLB6 *	AT3G61560.1	0.019%	0.170%		+	+		+	+
SYTA, SYT1 | synaptotagmin A *	AT2G20990.1	0.032%	0.081%		+	+		+	+
Root hair defective 3 GTP-binding protein (RHD3)	AT3G13870.1	0.019%	0.052%		+	+		+	+
DWF1, DIM, EVE1, DIM1, CSB1 | cell elongation protein	AT3G19820.1	0.023%	0.036%		+	+		+	+
DGL1 | dolichyl-diphosphooligosaccharide-protein glycosyltransferase *	AT5G66680.1	0.013%	0.023%	FRET-FLIM	+	+	2nd MS run	+	+
ATB5-B, B5 #3, ATCB5-D, CB5-D | cytochrome B5 isoform D	AT5G48810.1	0.013%	0.019%		+	+		+	+
SMT1, CPH | sterol methyltransferase 1 *	AT5G13710.1	0.003%	0.013%		+	+		+	+
CYP83B1, SUR2, RNT1, RED1, ATR4 | cytochrome P450, family 83	AT4G31500.1	0.010%	0.013%		+	+		+	+
PIP3, PIP3A, PIP2;7, SIMIP | plasma membrane intrinsic protein 3 *	At4G35100.1	0.010%	0.013%		+	+		+	+
Remorin family protein REM1.3 *	AT2G45820.1	0.010%	0.010%		+	+		+	+
Remorin family protein REM1.2 *	AT3G61260.1	0.013%	0.010%		+	+		+	+
FLA8, AGP8 | FASCICLIN-like arabinogalactan protein 8 *	AT2G45470.1	0.007%	0.010%		-	-		-	-
VAP27-1, VAP, (AT)VAP, VAP27 | vesicle associated protein	AT3G60600.1	0.007%	0.007%		+	+		+	+
ANNAT4 | annexin 4 *	AT2G38750.1	0.010%	0.007%		-	-		-	-
ATTRX3, ATH3, ATTRXH3, TRXH3, TRX3 | thioredoxin 3 *	AT5G42980.1	0.007%	0.003%		-	-		-	-
TCP-1/cpn60 chaperonin family protein *	AT3G03960.1	0.013%	0.000%		+	-		+	-

Table 1: Flowchart of the 17 proteins tested by FRET-FLIM with protein description (column 1) and accession number (column 2) indicating their % abundance in the total spectrum for RTNLB3 (column 3) or RTNLB6 (column 4), respectively. Proteins present in the PD proteome (Fernandez-Calvino et al., 2011) are marked with an asterisk. Positive (+) or negative (-) results for interaction in the FRET-FLIM analysis with either RTNLB3 (column 5) or RTNLB6 (column 6) are shown. The re-appearance of the corresponding protein in the second MS run is shown (RTNLB3-2 and RTNLB6-2, column 7 and 8).

Mass spectrometry confirmation of proteomics data

For further confirmation of the data, the GFP-Immunoprecipitation and MS proteomics was repeated with independent biological samples of RTNLB3-YFP and RTNLB6-YFP plants. This second dataset was then compared with data from the first experiment and only proteins present in both datasets compiled into a final list of interaction candidates.

This resulted in 42 interaction candidates for RTNLB3 and 57 for RTNLB6. Proteins were again ranked according to the quantity of peptide present in the total spectra. Interestingly proteins that were identified by FRET-FLIM to be false-positives in the first MS run (FLA8, ANNAT4 and TRX3) were not present in the second MS dataset thereby confirming and validating the FRET-FLIM methodology (Table 1).

Conclusions and Outlook

Our combined experimental approach of using sensitive pulldown assays coupled with FRET-FLIM provides a robust means of identifying functional interactions for reticulon proteins. The primary MS dataset was validated using FRET-FLIM and showed that more than 80% of the candidate proteins were indeed interacting with the reticulons. The intermediate dataset was confirmed by a second set of proteomics data for both reticulons and confirmed both the proteomics as well as in particular the FRET-FLIM analysis indicating a high confidence for the final protein interactome.

Using FRET-FLIM we are able to evaluate protein interactions in vivo with the proteins present at their native subcellular location and membrane topology.

Future aims include approaches for synthetic biology and protein complexes where protein locations and formations can be altered and changes in protein interaction studied.

Here we measured the interaction in the ER around the nuclear envelope but we have also been successful in detecting protein interactions within plasmodesmata which comprises a gap between cells of 30 nm and less.

Further future approaches might include measuring the interaction between multiple proteins such as metabolons, enzyme complexes that comprise all the enzymes needed for a complete biosynthetic pathway allowing for functional enhancement as well as synthetic formation of such enzymatic machineries.

Work published in:

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