

Chapter 2

Exploring the Protein Composition of the Plant Nuclear Envelope

Xiao Zhou, Kentaro Tamura, Katja Graumann, and Iris Meier

Abstract

Due to rather limited sequence similarity, targeted identification of plant nuclear envelope and nuclear pore complex proteins has mainly followed two routes: (1) advanced computational identification followed by experimental verification and (2) immunoaffinity purification of complexes followed by mass spectrometry. Following candidate identification, fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) provide powerful tools to verify protein–protein interactions in situ at the NE. Here, we describe these methods for the example of *Arabidopsis thaliana* nuclear pore and nuclear envelope protein identification.

Key words *Arabidopsis thaliana*, KASH protein, Nuclear pore complex, Bioinformatics, Immunoaffinity purification, Fluorescence resonance energy transfer (FRET), Fluorescence recovery after photobleaching (FRAP)

1 Introduction

The plant nuclear envelope (NE) proteome has remained fairly elusive until rather recently [1]. Due to limited sequence similarity of NE and nuclear pore complex (NPC) proteins, identification by sequence similarity alone has only revealed a handful of proteins [2–11]. Thus, computational approaches searching for patterns instead of sequences and reiterative complex purification coupled with mass spectrometry—starting with the few known proteins—have to date been the most successful strategies to identify plant NPC and NE proteins [12]. Once identified, candidates must be verified as members of NE and NPC complexes. Fluorescence recovery after photobleaching (FRAP) and fluorescence resonance energy transfer (FRET) complement biochemical methods to verify protein–protein interactions in situ at the NE.

KASH proteins are outer nuclear membrane (ONM) proteins that contain a single transmembrane domain (TMD) spanning the outer nuclear membrane followed by a short C-terminal KASH

domain that interacts in the perinuclear space with the SUN domain of SUN proteins [1, 13–16]. Although the N-terminal cytoplasmic domain of KASH proteins varies, the C-terminal KASH domain is relatively conserved, especially the C-terminal four amino acids [1, 13, 14, 17]. For example, the C-terminal four amino acids of animal KASH proteins can be summarized to a “[PATHQL]PP[QTVFILM]” motif (square brackets enclose alternative amino acid residues at the respective position), suggesting that a similar pattern could also be found in plants [18].

The program DORY was developed to search for a putative KASH (pKASH) domain that should (1) be immediately C-terminal of a TMD, (2) be short (less than 40 amino acids based on known KASH proteins), and (3) terminate in a given four-amino-acid pattern [18]. DORY contains two functional units—the KASHFilter and the HomologyFilter. The KASHFilter collects protein sequences that contain a pKASH domain and the HomologyFilter divides these protein sequences into homologous groups (Fig. 1). Proteins in each group potentially belong to one protein family.

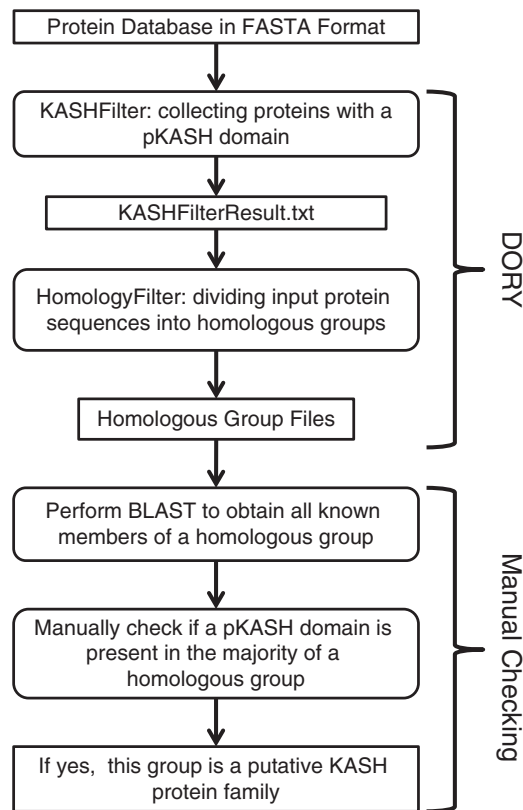


Fig. 1 Workflow of identifying KASH proteins using DORY. The steps can be divided into two sections—DORY search and manual checking. Input files, output files, and results are indicated by *rectangle frames*, and the searching steps are indicated in *round rectangular frames*

Considering that a protein sequence that is not a KASH protein may contain a pKASH domain by chance, we argue that if a pKASH domain is conserved in a protein family, then this protein family is more likely a functional KASH protein family. Therefore, the presence of a pKASH domain needs to be analyzed at the protein family level. Since DORY filters out proteins without a pKASH domain, researchers need to choose a protein sequence from each homologous group to collect all of its known homologs by BLAST and check if the pKASH domain is conserved in its homologs. The whole process is depicted in Fig. 1.

Although genome projects have provided large lists of genes, many gene products remain functionally uncharacterized. Determining the composition of protein complexes and the interaction networks in an organelle of interest establishes a framework, which generates strategies and hypotheses relating to the function, mechanism, and regulation of the organelle dynamics [19, 20]. We describe methods to isolate the *Arabidopsis* nuclear pore complex (NPC), which is one of the largest macromolecular protein complexes in the cell. By using immunoaffinity purification, NPC components are effectively purified from lysates of transgenic plants expressing GFP-tagged nucleoporins [12]. Subsequent mass spectrometry comprehensively identifies protein components in the affinity-purified complexes.

In the *Arabidopsis* genome, very few homologs of yeast and animal nuclear envelope proteins have been found. In such a case, the biochemical identification of protein complexes, which is independent of homology-based approaches, is useful. This approach is also a convenient and powerful method for revealing proteome-wide interactome maps that provide significant insights into functions of unknown proteins.

Fluorescence recovery after photobleaching (FRAP) is a live-cell imaging technique that enables the study of the mobile behavior of a protein and from this to draw conclusions about the functional properties of the protein. The protein under investigation needs to be fused to a fluorescent protein for confocal imaging. Low-power lasers are used to visualize the fusion proteins while causing as little damage as possible to the cells and tissue. The principle behind FRAP is that the laser not only excites the fluorophore, thus causing it to fluoresce and become visible, but at a higher output the laser causes irreversible structural damage to the fluorophore, which blocks the molecule from becoming excited. During FRAP, a selected area of fluorescence inside a cell is bleached followed by fluorescence recording. If fluorescence signal recovers in the selected area, excitable molecules have entered the area post-bleach, indicating protein movement (Fig. 2a).

Parameters measured and calculated include maximum fluorescence recovery (*MFR*), half time ($T_{1/2}$), mobile fraction, immobile fraction (Fig. 2b), and diffusion coefficient (*D*) [21].

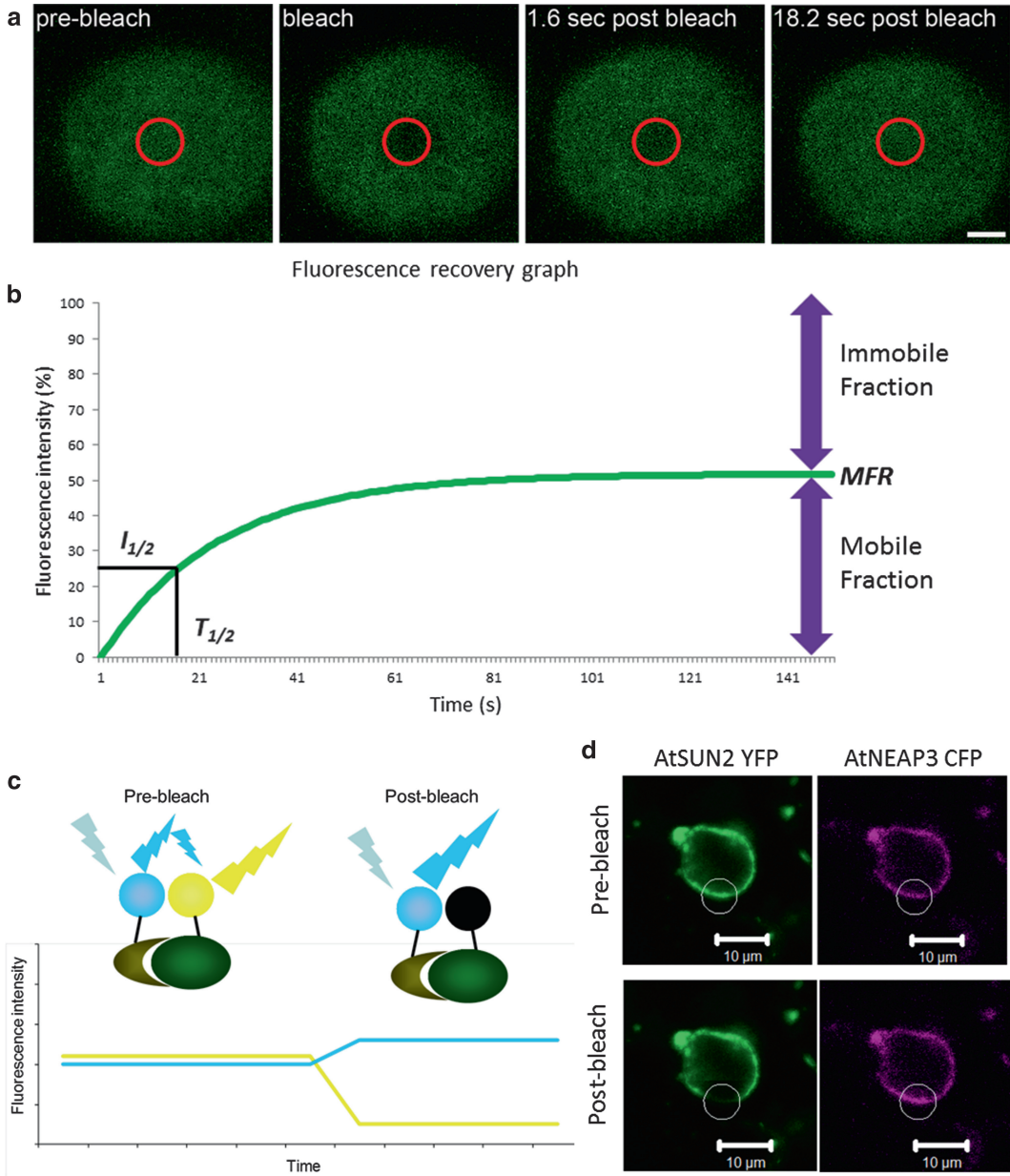


Fig. 2 Overview of FRAP and apFRET working concepts. **(a)** FRAP of LBR-GFP-labeled NE sheet; *red circle* is bleach ROI; fluorescence recovers quickly as LBR-GFP is highly mobile in NE membranes [27]. **(b)** Fitted fluorescence recovery curve (in this case of AtSUN1-YFP) displaying FRAP parameters typically analyzed. **(c)** Concept of apFRET; *green* structures are interacting proteins CFP and YFP are fused to; size of lightning indicates fluorescence intensity; the graph below shows fluorescence intensity of CFP (*blue line*) and YFP (*yellow line*) during apFRET; after the YFP bleach, YFP fluorescence diminishes and CFP fluorescence increases as YFP can no longer be excited with energy emitted by CFP. **(d)** Example of apFRET occurring between AtSUN2-YFP and AtNEAP3-CFP indicating interactions between the two NE proteins AtSUN2 and AtNEAP3 (Pawar, Evans, and Graumann, unpublished observations). YFP fluorescence in the white ROI diminishes after the bleach while CFP fluorescence in the ROI increases

The *MFR* is the highest post-bleach fluorescence intensity value and signifies the mobile fraction of a protein population. Subtraction of the mobile fraction from the pre-bleach fluorescence intensity value (100 %) results in the immobile fraction, the protein population that was bleached but not replaced by unbleached molecules. The assumption is that immobilized proteins are kept in place by binding interactions that anchor the proteins. Thus, *MFR*, mobile fraction, and immobile fraction are used to quantify mobile proteins. $T_{1/2}$ and D , in turn, are used to describe the quality of movement. $T_{1/2}$ is the time point at which half of the fluorescence has recovered. It is an indirect measure of protein velocity. D can be easily calculated if FRAP has been carried out in a two-dimensional structure such as the nuclear envelope (NE) sheet or an endoplasmic reticulum (ER) cisternae as it describes the protein movement more directly in $\mu\text{m}^2/\text{s}$ in the selected area. The velocity of protein movement can be affected by weaker binding interactions and by molecular obstacles hindering free protein movement. For membrane proteins, these hindrances can be caused by protein crowding of the membrane, lipid composition and density of the membrane, as well as structural networks interacting with the membrane and its embedded proteins [21]. These structural networks include the cell wall at the cell membrane [22] and the lamina at the NE [23]. FRAP has been used successfully to characterize NE proteins in various eukaryotic organisms and cell types [23–28].

Likewise, fluorescence resonance energy transfer or Förster resonance energy transfer (FRET) can be used in combination with live-cell confocal imaging and fluorescent protein fusions to study NE proteins. Specifically, FRET visualizes direct protein interactions in situ. It complements other protein interaction assays such as co-immunoprecipitation and yeast two-hybrid assays to confirm protein interactions in situ at the observed cellular location (in this case the plant NE).

In FRET, the emission energy of the donor fluorophore is used to excite the acceptor fluorophore (Fig. 2c). This transfer of energy can only occur when (1) the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor fluorophore overlap and (2) donor and acceptor fluorophores are in very close proximity—1 to 10 nm [29–31]. The two compatible fluorophore pairings for FRET commonly used are GFP–mRFP and CFP–YFP, where GFP and CFP are the donors and mRFP and YFP are the respective acceptors (Fig. 2c, d). The distance between the two fluorophores is affected by protein interactions but also protein folding and localization. Donor and acceptor are fused to two proteins, whose interaction can bring donor and acceptor into sufficiently close proximity for FRET to occur. However, this depends on the conformation and compartmentalization of the protein pair. For instance, SUN and KASH domains

interact with each other [1, 14]. However, if the SUN protein contains the fluorophore at its nucleoplasmic N-terminus and the KASH protein contains the fluorophore on its cytoplasmic N-terminus, then the interaction is not observed by FRET because the distance between the two fluorophores is too great for energy transfer to occur.

FRET can be carried out in several ways—this chapter will focus on acceptor photobleaching FRET (apFRET), in which the acceptor fluorophore is bleached as described for FRAP. By bleaching the acceptor fluorophore, it can no longer use the donors' emission energy for excitation, which results in the donor emission fluorescence increasing [29, 31]. Hence, the donor fluorescence is monitored before and after the acceptor is bleached. If the donor fluorescence increases after the acceptor bleach, the two proteins fused to the donor and acceptor are considered to interact with each other (Fig. 2c, d). The difference in donor fluorescence before and after acceptor bleach is termed the FRET efficiency (E_F) and is expressed as a percentage. The E_F does not give indications on the strength of the interaction, i.e., a high E_F does not mean a strong binding interaction as the E_F is primarily dependent on the distance between the donor and acceptor fluorophore and their spectral overlap [31]. The apFRET technique has been successfully used to demonstrate protein interactions for both soluble and membrane-bound proteins in various experimental systems. Karpova et al. [29] provide a comprehensive description of apFRET to investigate binding of soluble nuclear proteins. In plant NE biology, apFRET has been used to study SUN proteins [25, 26, 32]. FRAP and apFRET techniques will be considered separately below. For apFRET, the conditions described will be for the donor–acceptor pairing YFP–CFP.

2 Materials

2.1 Computational Identification of Plant KASH Proteins

1. DORY: This program can be downloaded from the supplemental files of Zhou et al. [18] or from the following link: <http://sourceforge.net/projects/doryforkash/>.
2. Computer specifications: DORY is written in Java, and therefore a personal computer with Java installed is needed. A 64-bit computer system and 64-bit Java are recommended.
3. Jalview: a multiple sequence alignment analysis tool that can be downloaded at http://www.jalview.org/Web_Installers/install.htm.
Internet connection is required because the following online resources will be needed:
4. BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).
5. Phobius (<http://phobius.sbc.su.se/>).

6. MAFFT (<http://mafft.cbrc.jp/alignment/server/>).
7. Protein database file: This must be in FASTA format. DORY can only read FASTA format files and does not check the input file format. To generate this file, the NCBI nonredundant protein sequences used in BLAST can be downloaded at <ftp://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/nr.gz>.

2.2 Proteomic Identification of New Plant Nuclear Envelope and Nuclear Pore Proteins

2.2.1 Immuno-precipitation

1. Lysis buffer: 50 mM Hepes–KOH pH 7.5, 150 mM NaCl, 0.5 % (v/v) Triton X-100, 0.1 % (v/v) Tween-20. Store at 4 °C.
2. Wash buffer: 50 mM Hepes–KOH pH 7.5. Store at 4 °C.
3. Elution buffer: 100 mM Tris–HCl pH 6.8, 2 % (w/v) SDS, 20 % (w/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 0.005 % (w/v) bromophenol blue.
4. Matrix for pulldowns conjugated to anti-tag antibody. This protocol has been optimized for magnetic beads conjugated to an anti-GFP antibody, specifically μ MACS Anti-GFP MicroBeads (Miltenyi Biotec, Germany) (*see Note 1*).
5. Support materials for pulldowns, e.g., the μ MACS system requires μ Columns and μ MACS Separator (both from Miltenyi).
6. Mortar and pestle.
7. Liquid nitrogen.

2.2.2 SDS-PAGE and Flamingo Staining

1. Running gel buffer: 1.5 M Tris–HCl pH 8.8.
2. Stacking gel buffer: 1 M Tris–HCl pH 6.8.
3. Ammonium persulfate: 10 % (w/v) solution in water. Make aliquots and store at –20 °C (*see Note 2*).
4. *N,N,N,N'*-tetramethylethylenediamine (TEMED). Store at 4 °C.
5. SDS-PAGE running buffer: Dissolve 3.03 g Tris, 14.41 g glycine, and 100 g SDS in 1 L water.
6. Fixing solution: 40 % (v/v) ethanol, 10 % (v/v) acetic acid (*see Note 3*).
7. Gel staining solution: We use the Flamingo fluorescent gel stain (Bio-Rad, USA) for which 1 volume of the stock should be diluted with 9 volumes of water (*see Note 4*).

2.2.3 Plant Materials

1. Seven- to ten-day-old transgenic *Arabidopsis* seedlings (fresh weight 0.3 g–3 g) expressing GFP-tagged nucleoporin (*see Note 5*).
2. Seven- to ten-day-old transgenic *Arabidopsis* seedlings (fresh weight 0.3 g–3 g) expressing free GFP as a negative control (*see Note 5*).

2.2.4 Trypsin Digestion for MS Analysis

1. Acetonitrile (mass spectrometry quality reagent).
2. Ammonium bicarbonate, 100 mM stock solution.
3. Reducing buffer: 10 mM DTT in 50 mM ammonium bicarbonate (*see Note 6*).
4. Alkylating buffer: 55 mM iodide acetamide in 50 mM ammonium bicarbonate (*see Note 7*).
5. Trypsin solution: 0.01 mg/mL trypsin in 50 mM ammonium bicarbonate. A high grade is required for MS analysis; we use “sequence grade” (Promega, USA). Make aliquots and store at -20°C (*see Note 8*).
6. Peptide extraction buffer: 5 % (v/v) formic acid in 50 % (v/v) acetonitrile.

2.3 Imaging Techniques to Identify Protein–Protein Interactions at the Plant Nuclear Envelope

2.3.1 FRAP

1. Plant material expressing a NE protein fused to GFP or its variants CFP and YFP (*see Note 9*). Plant material can be either stably or transiently expressing and can be from any tissue that is normally easy to image (e.g., leaf, root, anthers) or cell culture (e.g., BY-2 cells).
2. Confocal microscope with laser, filter, beam splitter, and channel settings to image GFP, CFP, or YFP (Table 1).
3. Mounting materials including razor blade, pipette, microscope slide, cover slip, water, and lens oil (if oil-dipping lens used; *see Note 10*).
4. Microsoft Excel and GraphPad Prism for data analysis (*see Note 11*).

2.3.2 apFRET

1. Plant material co-expressing two NE proteins: one fused to CFP and the other to YFP. Plant material can be either stably or transiently expressing and can be from any tissue that is normally easy to image (e.g., leaf, root, and anthers) or cell culture (e.g., BY-2 cells).
2. Plant material expressing only the CFP-fused protein as control. Carry out the same apFRET experiment with CFP only

Table 1
Beam path settings for imaging GFP, CFP, and YFP with a Zeiss LSM confocal microscope

Fluorophore	Excitation laser wavelength (nm)	Emission wavelength captured (nm)	Beam splitters and filters for Zeiss LSM
GFP	488	505–530	HFT488
CFP ^a	458	470–500	HFT458/514; NFT515
YFP ^a	514	530–600	HFT458/514; NFT515

^aSettings for simultaneous imaging of CFP and YFP

expressing tissue to ensure that an increase in CFP fluorescence in the co-expressing sample is due to the YFP bleach and hence protein interactions. Changes in CFP fluorescence when only CFP is expressed can be due to laser fluctuations, sample drifting, or membrane/organelle movement.

3. Confocal microscope with laser, filter, beam splitter, and channel settings that enable the simultaneous imaging of CFP and YFP (Table 1).
4. Mounting materials including razor blade, pipette, microscope slide, cover slip, water, and lens oil (if oil-dipping lens used; *see Note 12*).
5. Microsoft Excel for data analysis (*see Note 11*).

3 Methods

3.1 Computational Identification of Plant KASH Proteins

DORY has a user-friendly interface and can be easily set up. In the following text, the parameters adjustable in DORY will be in *italic*.

3.1.1 Setting Up “KASHFilter” Parameters

1. Set up “*TMD Frame Length*” and “*TMD Hydrophobic Threshold*” to identify proteins with a single TMD. DORY identifies potential TMDs by reading an amino acid sequence using a frame with a certain amino acid number (can be set in “*TMD Frame Length*”), sums up the hydrophobic value of each amino acid in this frame, and compares the sum with the “*TMD Hydrophobic Threshold*.” If the sum is not less than the “*TMD Hydrophobic Threshold*,” then the sequence in the frame is considered a TMD. The default values of “*TMD Frame Length*” and “*TMD Hydrophobic Threshold*” (20 and 32, respectively) have been tested to work best for identifying TMDs. Proteins with a single TMD will be kept, and the sequence C-terminal of the TMDs (the tail) will be subjected to further analysis (see next step).
2. Set up “*Maximum KASH Tail Length*,” “*Minimum KASH Tail Length*,” and “*Regex for KASH Tail*” to identify pKASH domains. If the length of the tail from the previous step is not more than “*Maximum KASH Tail Length*” and not less than “*Minimum KASH Tail Length*,” then it will be determined whether this tail terminates in a given C-terminal four-amino-acid pattern. This is done by comparing the tail to a regular expression set in “*Regex for KASH Tail*.” The C-terminal four-amino-acid pattern can be summarized from known KASH proteins, and some presets can be chosen from the dropdown menu of the “*Regex for KASH Tail*.” To customize “*Regex for KASH Tail*,” knowledge of regular expression is needed, and

the details can be found at <http://www.regular-expressions.info>. Some basics of regular expression are explained below.

Symbol	Match
\S	Any non-space characters
\S+	One or multiple non-space characters
[...]	Any character inside the square brackets
\Z	The end of a sequence

If the tail passes the regular expression test, it is considered as a pKASH domain and the protein sequence will enter an output file called “KASHFilterResult.txt.”

If “*Output potential KASH tail in a file during the KASHFilter search*” is checked, then the pKASH domain will be output to a file named “KASHTail.txt.” If “*In the output file, left pad KASH tail to the Maximum KASH Tail Length*” is also checked, the output pKASH domain sequences will be right aligned.

3. To confine a search to proteins within an amino-acid-length range, use the “*Protein Length Cutoff from to*” parameters.
4. If the protein names in the database contain species names, there are two ways to confine a search to proteins that belong to certain species.
 - (a) Check the “*During KASHFilter search, keep the proteins whose protein names contain:*” checkbox. Click “*Choose Species Name File (one line one name)*” and choose a text file containing species names. In this text file, each line should contain only one species name. DORY will load the species names in this text file and simply check whether a protein name contains any of these species names. If yes, then this protein will be kept for further analysis; otherwise, it will be ignored.
 - (b) Use “*Query NCBI Taxonomy Browser to filter non-eukaryotic proteins out.*” This is specifically designed for the NCBI nonredundant protein sequences file. DORY will read out the species name from the name of a protein and send a request to the NCBI Taxonomy server. If the response text contains the text set in “*Being positive, server return text should contain*” textbox, then this protein will be kept for further analysis; otherwise, it will be ignored.

3.1.2 Setting Up “HomologyFilter” Parameters

The KASHFilter will generate a file containing proteins with pKASH domains(KASHFilterResult.txt). Then the HomologyFilter can read this file, group the proteins into homologous groups, and save each group in separated text files with numbered names.

HomologyFilter uses two parameters, the “*E-value Cutoff*” and the “*Homolog Cutoff*.”

1. “*E-value Cutoff*.” Two proteins are considered homologs if the E-value of aligning these two proteins is less than the “*E-value Cutoff*” (see Subheading 3.1.5, **step 2**). DORY calculates the E-value using $Kmne^{-\lambda S}$. S is the score of an alignment using the Smith–Waterman algorithm. K and λ are Karlin–Altschul parameters whose values are obtained from the BLAST source code. Parameters m and n are the effective lengths of the query sequence and database, respectively. They are calculated by a modified “BLAST_ComputeLengthAdjustment” function from the BLAST source code.
2. “*Homolog Cutoff*.” If an output group contains homologs less than the “*Homolog Cutoff*” value, then output files will be labeled with the prefix “belowHomoCutOff.”

3.1.3 Setting Up Running Parameters

Either a full search can be performed, or only the KASHFilter can be run. A KASHFilterResult.txt file from a previous run of the KASHFilter can be directly run with the HomologyFilter, but the “*Database Total Protein Length*” and “*Database Total Protein Number*” have to be provided, which can be found in the log file of the previous run of the KASHFilter.

3.1.4 Running DORY

1. Click “*Open Database File*” button and choose a database file.
2. Click the “*Run*” button.
3. DORY will create a folder in the directory of the database file.
4. Inside this folder DORY will output the results and a log file that documents all parameters and steps of this run (see **Note 14**, if DORY takes too long to finish).

3.1.5 Manual Confirmation of the Candidates

After DORY outputs the homologous groups, the user must manually check whether a pKASH domain is present in the majority of a homologous group (see **Note 15**).

1. Choose one or more proteins from each group and perform protein BLAST against the nonredundant protein sequences. Since KASH proteins are eukaryotic proteins, the “*Organism*” parameter can be set to “*Eukaryota*.” “*Expect threshold*” should be set to $1e-4$ or lower. However, if the threshold is too low, some true homologs will be filtered out. “*Max target sequences*” can be set at 500 at first and increased to a higher number if the maximum target sequence number is reached at the first round of BLAST. Click the “*BLAST*” button to run.
2. In the result webpage of BLAST, click “*All*” in the “*Select*” section. Manually uncheck undesired sequences if necessary (see **Note 13**). In the “*Download*” dropdown menu, choose

“FASTA (complete sequence)” to obtain the protein sequences of the selected homologs. The homologs identified by DORY and BLAST depend on the E-value threshold. DORY and BLAST use local alignment methods, which means that proteins which contain domains homologous to parts of the query protein may pass the E-value threshold. For example, Nesprin-1 contains actin-binding domains and spectrin repeats. Proteins containing any of these two domains may be classified as “homologs.” However, they may not belong to the same protein family. Therefore, a large homologous group output by DORY may need to be further analyzed, especially when it contains large proteins having multiple domains.

The following steps are to determine whether a homologous group output by DORY belongs to a single protein family. Before manually performing the following analysis steps, it is worth trying the HomologyFilter again with a more stringent “E-value Cutoff.”

- (a) Use MAFFT to align the protein sequences of a homologous group. Set “Output order” to “Aligned” before starting the alignment.
 - (b) Download the alignment in “Clustal format,” and open the alignment in Jalview.
 - (c) In Jalview menu, choose “ClustalX” in the “Colour” menu, uncheck “Wrap” in the “Format” menu, and adjust font in the “Format” menu to obtain an overview of the alignment.
 - (d) Scroll to manually check whether the sequences can be divided into sub-homologous groups based on the alignment.
 - (e) If yes, choose one sequence in a group by clicking it. Choose “Remove All Gaps” in the “Edit” menu. Then right-click, and choose “Selection”->“Edit”->“Copy.” Paste the sequence in the BLAST webpage and set up a BLAST search. After this, in Jalview, choose “Undo Remove Gaps” to restore the alignment. Do this for all the sub-homologous groups identified. Compare the BLAST results of these subgroups to assess whether they belong to the same homologous group. If multiple homologous groups are identified, perform the next step for each one, respectively.
3. The presence of a C-terminal TMD can be tested using Phobius [33]. Phobius is also a prediction program and its prediction may not be accurate for a particular homolog, but it will provide an overview of whether a C-terminal TMD is predicted in the majority of a homologous group. If a C-terminal TMD is predicted, then check whether the C-terminal four amino acids

of the majority of this homologous group follow the pattern set in “*Regex for KASH Tail*.” If yes, then this homologous group is probably a KASH protein family within the larger superfamily of KASH domain-containing proteins. It is noteworthy that not all of the proteins in a homologous group will terminate in four amino acids exactly following the pattern. For example, when using the preset C-terminal four-amino-acid pattern “[PATHQL]PP[QTVFILM]” to identify animal KASH proteins, in the homologous groups obtained by BLAST, proteins terminating in PLPV and PSPT can also be found. “PLPV” and “PSPT” are quite similar to the known KASH domain C-terminal four-amino-acid patterns “PPPV” and “PPPT,” respectively. Therefore, these outliers are probably also KASH proteins, and their C-terminal four amino acids can be used to improve the pattern used in “*Regex for KASH Tail*” (*see below*).

3.1.6 Improve the “*Regex for KASH Tail*”

After the manual confirmation, a new pattern of the C-terminal four amino acids may be summarized from the proteins believed to be KASH proteins. This new pattern can be used as an improved “*Regex for KASH Tail*” to perform a new round of searching.

3.1.7 Use DORY for Other Purposes

DORY can also be used to identify proteins that contain one TMD followed by a short conserved C-terminal sequence, as long as the conserved C-terminal sequence can be summarized by a generalized consensus. The steps to do this are the same as identifying KASH proteins, except with replacing the consensus sequence in the search parameters. In addition, with the source code available, DORY can be modified to perform searches according to individual needs.

3.2 Proteomic Identification of New Plant Nuclear Envelope and Nuclear Pore Proteins

3.2.1 Immuno-precipitation with Anti-GFP Antibody Beads

Carry out all procedures at 4 °C to protect from protein degradation unless otherwise specified.

1. Grind *Arabidopsis* seedlings to a powder in liquid nitrogen with a mortar and pestle.
2. Add ice-cold lysis buffer (3× volumes/gram fresh weight) to the powder and mix well. Transfer the lysates to a 1.5 mL tube and centrifuge the lysates at 20,400 × *g* for 5 min to remove cellular debris.
3. Transfer the supernatant to a new tube and centrifuge at 20,400 × *g* for 5 min to remove cellular debris completely.
4. Transfer the supernatant to a new tube and add 50 µL of magnetic beads conjugated to an anti-GFP antibody (e.g., µMACS Anti-GFP MicroBeads). Incubate for 30 min on ice.
5. Place µColumn in the magnetic field of the µMACS Separator. Equilibrate the column by applying 200 µL of lysis buffer.

6. Apply the lysates onto the column and flow through by gravity.
7. Rinse the column with $4 \times 200 \mu\text{L}$ lysis buffer.
8. Rinse the column with $1 \times 100 \mu\text{L}$ wash buffer (*see Note 16*).
9. Apply $20 \mu\text{L}$ of preheated 95°C hot elution buffer to the column and incubate for 5 min at room temperature. Apply $50 \mu\text{L}$ of preheated 95°C hot elution buffer to the column and collect eluate as the immunoprecipitate.

3.2.2 SDS-PAGE and Flamingo Staining

1. Prepare a 12.5 % gel for SDS-PAGE: Mix 5 mL of running gel buffer, 8.3 mL of 30 % acrylamide/bis mixed solution (29:1), 0.2 mL of 10 % SDS, 3.8 mL of water, and 0.2 mL of ammonium persulfate (*see Note 17*). Add $8 \mu\text{L}$ of TEMED to begin polymerization of acrylamide gel and cast gel within a $7.5 \text{ cm} \times 15 \text{ cm} \times 1 \text{ mm}$ gel cassette.
2. Mix 0.375 mL of stacking gel buffer, 1 mL of 30 % acrylamide/bis mixed solution, $60 \mu\text{L}$ of 10 % SDS, 4.475 mL of water, and $60 \mu\text{L}$ of ammonium persulfate. Add $6 \mu\text{L}$ of TEMED and gently pour into gel caster. Insert a gel comb immediately without introducing air bubbles. Incubate at room temperature for 30 min to allow polymerization.
3. Apply the samples and electrophorese at 30 mA until bromophenol blue dye front from the elution buffer has reached the bottom of the gel.
4. Place the gel in a clean tray with 200 mL of fixing solution and incubate with gentle agitation for at least 2 h (*see Note 18*).
5. Pour off fixing solution and add 200 mL of Flamingo staining solution. Cover the gel tray with aluminum foil to limit light exposure and incubate with gentle agitation for at least 3 h (*see Note 19*).
6. Image the stained gels with fluorescent laser light (470–530 nm) and a longpass emission filter. Excise the protein bands of interest and transfer to a 1.5 mL tube (*see Note 20*).

3.2.3 In-Gel Digestion and Peptide Extraction for Mass Spectrometry

1. Wash the gel bands twice with $200 \mu\text{L}$ of 25 mM ammonium bicarbonate in 30 % (v/v) acetonitrile for 10 min followed by 100 % (v/v) acetonitrile for 15 min. Dry in a vacuum concentrator.
2. Add $200 \mu\text{L}$ of reducing buffer and incubate with shaking at 56°C for 45 min.
3. Remove reducing buffer from the tube and add $200 \mu\text{L}$ of alkylating buffer. Incubate in dark at room temperature for 30 min.
4. Wash the gel bands with $200 \mu\text{L}$ of 50 mM ammonium bicarbonate followed by three times with $200 \mu\text{L}$ of 50 % (v/v)

acetonitrile in 50 mM ammonium bicarbonate. Dry in a vacuum concentrator.

5. Add 20 μL of trypsin solution and incubate at 37 °C overnight.
6. Recover the digested peptide twice with 20 μL of 5 % (v/v) formic acid in 50 % (v/v) acetonitrile. Combine the extracted peptide solutions and evaporate to 10 μL in a vacuum concentrator (*see Note 20*).
7. Subject the digested peptide to mass spectrometry.

3.3 Imaging Techniques to Identify Protein–Protein Interactions at the Plant Nuclear Envelope

3.3.1 FRAP

1. Set up the microscope for a FRAP experiment. Use a 100 \times or 63 \times oil or water dipping lens and minimal digital zoom factor. Keep laser transmission low, typically at 1–10 % to avoid photobleaching while measuring fluorescence recovery (transmission settings are dependent on microscope and fluorophore). Set up appropriate imaging setting for fluorophore (Table 1) and select a region of interest (ROI), in which fluorescence will be bleached and recovery measured. Select a control ROI, which is not bleached to monitor stability of fluorescence during the FRAP experiment. Keep the size of the ROI between different samples constant.
2. Set up bleaching options for bleach ROI: at least 5 pre-bleach scans to establish average pre-bleach fluorescence followed by bleach and post-bleach scans. The bleach should be carried out with the laser transmission set at 100 %, and the number of bleaching iterations is dependent on the strength of the signal; start at 2–3. The number of scans depends on the time period fluorescence recovery will be observed for. Diffusion is a fast process, which occurs in a matter of seconds, whereas protein turnover occurs over minutes and hours. If a long time course is selected, keep larger time intervals between post-bleach scans so as not to bleach recovering fluorescence. Keep bleach parameters between different samples constant. Use the same pinhole for bleach and recovery.
3. Mount plant tissue by excising the tissue with a razor blade and mounting it in water on a microscope slide. Alternatively, mount cultured cells in their culture medium on a microscope slide. Place the cover slip, and if necessary seal it on the slide with either double-sided tape or Valap (*see Notes 10, 12, and 21*).
4. Image the tissue to find appropriate nuclei. Before carrying out the FRAP experiment, choose whether to bleach the NE sheet (necessary if D needs to be calculated) or the NE rim in the nuclear midsection.
5. Place the bleach ROI over the area to be bleached and a control ROI over a fluorescence area not to be bleached. Carry out the bleach with settings defined in **step 2**.

6. Observe fluorescence intensity values in both ROIs over time. Typically, we take images every 1 s over a total period of 90 s for plant NE proteins. However, if proteins move more rapidly, shorter time periods and images per second can be used. Vice versa, slower movement (such as import) requires observations over longer time periods, and so, to avoid photobleaching, the time between each scan may be longer (*see step 2*).
7. If the fluorescence intensity stays constant in the control ROI, no uncontrolled bleaching has occurred and fluorescence values of the bleach ROI can be saved as a text file for subsequent analysis.
8. For each sample, carry out at least 30–50 bleach experiments and do not “reuse” the same nucleus.
9. Export raw fluorescence intensity values into an Excel file. In order to allow analysis and comparison of all samples, fluorescence intensity values need to be normalized to a percentage scale using the following formula:

$$I_N = \left[(I_T - I_{\text{MIN}}) / (I_{\text{MAX}} - I_{\text{MIN}}) \right] \times 100$$

where I_N is the normalized fluorescence, I_T is the fluorescence intensity at a given time point, I_{MIN} is the fluorescence intensity immediately after the bleach, and I_{MAX} is the average pre-bleach fluorescence intensity [27].

10. Export the normalized fluorescence intensity values for each sample into GraphPad Prism or a similar curve fitting software. Fit the data with a nonlinear regression. Useful equations in the GraphPad Prism library include one-phase association, one-site binding, and one-phase exponential association, but user-defined equations can also be used. Use the equation with the best fit (highest R^2 value). Use the fitted values to plot the recovery curve graph. The fitted value for the last time point is the *MFR*.
11. Calculate $T_{1/2}$ by dividing the *MFR* by 2 ($I_{1/2}$). In GraphPad Prism, use $I_{1/2}$ to interpolate $T_{1/2}$. Alternatively, the curve equation can be used to calculate $T_{1/2}$ manually by using $I_{1/2}$.
12. Calculate D if fluorescence recovery was measured in a two-dimensional structure (e.g., the NE sheet). Use the following equation for this:

$$D = 0.88 \times (\omega^2 / 4 \times T_{1/2})$$

where ω is the radius of the bleach ROI [34].

13. Determine the average *MFR*, $T_{1/2}$, and, if calculated, D from the approximately 30–50 bleach experiments of each data set and carry out statistical analysis of these parameters. These can typically include F-tests for variance and Student's t -test for differences between data sets.

3.3.2 *apFRET*

1. Set up the microscope for an apFRET experiment. Use a 100× or 63× oil or water dipping lens and a minimal digital zoom factor. Keep laser transmission low, typically at 1–10 % to avoid uncontrolled photobleaching/activation of the acceptor and donor fluorophores. It is also wise to avoid white light on the samples as this can activate background FRET. Set up appropriate imaging settings for CFP and YFP (Table 1) and select a ROI in which acceptor fluorescence will be bleached and donor fluorescence will be measured. Keep the size of the ROI between different samples constant.
2. Set up bleaching options for YFP bleach: at least 5 pre-bleach scans to establish average pre-bleach fluorescence followed by bleach and post-bleach scans. Only one post-bleach scan is necessary. The bleach should be carried out with the YFP excitation laser transmission set at 100 %, and the number of bleaching iterations is dependent on the strength of the signal; start at 2–3. Keep bleach parameters between different samples constant.
3. Mount plant tissue by excising the tissue with the razor blade and mounting it in water on the microscope slide. Alternatively, mount cultured cells in their culture medium on the microscope slide. Place the cover slip and if necessary seal it on the slide with either double-sided tape or Valap (*see* **Notes 10, 12, and 21**).
4. Image the tissue to find appropriate nuclei.
5. Place the bleach ROI over the area to be bleached. Carry out the bleach with settings defined in **step 2**.
6. Observe the *first* fluorescence intensity values of both YFP and CFP *immediately after the bleach*. The YFP value should have significantly decreased (significantly compared to non-bleach YFP changes due to laser fluctuations), and the CFP fluorescence intensity may have changed after the bleach depending on interactions (to determine this, see steps below).
7. Fluorescence values of the bleach ROI can be saved as text file for analysis.
8. For each sample, carry out at least 30–50 bleach experiments and do not “reuse” the same nucleus. For analysis, only use the CFP fluorescence intensity values.
9. Export the raw CFP fluorescence intensity values into Excel. In order to allow analysis and comparison of all samples, fluorescence intensity values need to be normalized to a percentage scale using the following formula:

$$I_N = [(I_T - I_{MIN}) / (I_{MAX} - I_{MIN})] \times 100$$

where I_N is the normalized fluorescence, I_T is the fluorescence intensity at the given time point, I_{MIN} is the fluorescence intensity immediately after the bleach, and I_{MAX} is the average pre-bleach fluorescence intensity [27].

10. Calculate the average pre-bleach fluorescence intensity value for each sample.
11. For each sample, subtract the average pre-bleach fluorescence intensity value from the CFP fluorescence intensity value immediately after the bleach. The resulting value equals E_F .
12. Calculate the difference between two pre-bleach values to determine a no-bleach control E_F . In addition, carry out apFRET for the CFP only expressing sample and calculate the E_F value as a CFP only control E_F .
13. Calculate the average E_F , no-bleach control E_F and CFP only control E_F from the 30–50 experiments per data set and carry out statistical analysis. These can typically include F-tests for variance and Student's t -test for differences between controls and non-controls. If the two control (no YFP bleach and CFP only) E_F values are significantly lower than the no-control E_F value, the CFP fluorescence intensity has increased because FRET has occurred and the two fusion proteins interact with each other.

4 Notes

1. Miltenyi provides several different types of magnetic beads for other epitope tags.
2. Do not freeze/thaw more than twice.
3. Prepare just before using.
4. Prepare just before using.
5. Weight of plant material depends on the expression level of the GFP-fusion protein. In case of using the constitutive 35S promoter for expressing GFP fusions, 0.3 g of *Arabidopsis* seedlings is sufficient for this experiment.
6. Prepare just before using.
7. Prepare just before using.
8. Do not freeze/thaw more than twice.
9. mRFP can be used but bleaching efficiency of the mRFP laser is low due to the longer wavelength. GFP and its variants are advisable to use.
10. To avoid drifting of cells under the microscope, once the sample has been mounted, the slide can be sealed with either Valap or double-sided tape. Valap is made from equal parts Vaseline, lanolin, and paraffin wax.

11. OpenOffice or other curve fitting software might be used instead.
12. The nucleus can be quite mobile and might drift out of focus during the FRAP and apFRET experiments. To avoid nuclear movement, tissue sections can be treated with latrunculin B before mounting. For this, excise tissue and submerge in 25 μ M latrunculin B. Incubate for approximately 20–30 min (for leaf tissue) and then mount tissue as described. Note that this should not be used when interactions/protein mobility in connection with actin are examined.
13. The same issue needs to be taken into consideration when obtaining homologs of a protein using BLAST. The “Distribution of Blast Hits on the Query Sequence” section in the BLAST result webpage needs to be consulted. Only the protein sequences that have good whole-sequence alignment should be chosen to download.
14. DORY takes too long to finish. The workload of the HomologyFilter increases exponentially with the number of input proteins. If DORY takes too long to finish, it is very likely that the result of the KASHFilter contains too many protein sequences. In this case, stop DORY, refine the parameters of the KASHFilter, and try again.
15. Reasons that not all members of a putative KASH protein family contain a pKASH domain: First, protein sequences predicted from DNA sequences may have miss-predicted C-termini. Second, partial proteins may lack their C-termini. Third, splice variants of some KASH genes do not encode a KASH domain, for example, *Caenorhabditis elegans* zyg-12 [35]. Therefore, if the majority of a homologous group contains a pKASH domain, then this group can be considered as a putative KASH protein family.
16. To eliminate the possibility of interference of SDS-PAGE, it is an important step to remove high concentrations of residual salts and detergents from the immune complex before the elution step.
17. The final concentration of acrylamide here is 12.5 %; however, this can be varied according to the size of the proteins of interest.
18. Gels can be left in fixing solution for up to 24 h. Shortened fixation time may reduce sensitivity.
19. Gels can be stored in Flamingo staining solution for up to 6 months and imaged without significant loss of sensitivity. For long-term storage, the gels should be placed in the dark at 2–8 °C.

20. We highly recommend using the low-binding tubes and tips that ensure best recovery rates of the peptides.
21. If a long time course is used, plant material may deteriorate on the microscope slide. Use culture dishes or ½ MS agar on slides.

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