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Detecting the interaction of peptide ligands with plant membrane receptors

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Abstract

The field of plant receptor biology has rapidly expanded in recent years, however the demonstration of direct interaction between receptor-ligand pairs remains a challenge. Click chemistry has revolutionized small molecule research but lacks popularity in plant research. Here we describe a method that tests for the direct physical interaction of a candidate receptor protein and a peptide ligand. This protocol describes the generation of the ligand probe, transient expression of a receptor protein, enrichment of membrane-bound receptors, photo-crosslinking and click chemistry-mediated reporter addition, and detection of the receptor-ligand complex. Copper-based click chemistry confers several advantages, including the versatility to use almost any azide-containing reporter molecule for detection or visualization of the complex and addition of the reporter molecule after receptor-ligand binding which reduces the need for bulky ligand modifications that could interfere with the interaction.

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Plant receptor; ligand; click chemistry; photo-crosslinking

INTRODUCTION

This unit describes a procedure for observing direct physical interaction between a candidate receptor protein and its putative peptide ligand. Basic Protocol 1 describes the conjugation of a free peptide ligand to a reporter scaffold that consists of a trifluoromethyl-aryl diazirine moiety for photo-crosslinking and an alkyne handle for facile conjugation of reporter molecules via copper-based click chemistry. If solubility of the peptide is a problem, Alternative Protocol 1 details an on-bead strategy for ligand probe synthesis. Basic Protocol 2 explains how to generate large amounts of the affinity-tagged candidate receptor protein using transient overexpression in *Nicotiana benthamiana* and its subsequent isolation from plasma membrane-enriched microsomes. This step is essential for enriching the receptor proteins and removes UV-light-absorbing pigments that might affect the subsequent photo-crosslinking step. Finally, Basic Protocol 3 explains methods for the formation and detection of covalently-linked receptor-ligand complexes. After UV light is used to crosslink the ligand to its cognate receptor, the complex is captured on an affinity resin by targeting an affinity tag present on the candidate receptor. Subsequent ‘clicking’ of a reporter molecule (such as an affinity tag or fluorescent group) to the alkyne handle enables detection of the complex using standard PAGE and affinity blotting detection procedures. Reporter molecule addition after binding prevents it from interfering with ligand binding to the receptor. The chemical synthesis of the ligand-based probe takes about two weeks but will produce enough material for many assays. The remaining procedure takes about 9–13 days to complete (see Figure 1).

STRATEGIC PLANNING

Synthetic chemistry considerations

Ligand probe synthesis requires acquisition of both the peptide ligand (see below) and the trifluoromethyl-aryl diazirine alkyne acid (Figure 2). Because the trifluoromethyl-aryl diazirine compound is not currently available commercially, the user must either synthesize it themselves [using the published protocol by (Mayer & Maier, 2007)], collaborate with a laboratory with expertise in chemical synthesis, or contract with a synthesis core facility or contract research organization (CRO). An estimate of \$7000–\$8000 was given for custom synthesis of 500 mg of this compound by the Chemical Synthesis & Biology Core Facility at Albert Einstein College of Medicine, but cost estimates might vary between facilities. It is recommended to obtain the expertise of a synthetic chemist for this endeavor as the generation of trifluoromethyl-aryl diazirine will not be possible without the appropriate equipment and the expert knowledge found in a synthetic chemistry laboratory.

Ligand probe synthesis considerations

Custom peptides typically take a month to obtain from vendors so 1–5 mg of the peptide should be purchased as one of the first steps in preparation for this protocol. If non-target

primary amines, such as lysine residues, are present in the peptide sequence, they should be protected prior to conjugation with the crosslinking moiety, and deprotected after successful conjugation. This way, only the *N*-terminus on the peptide is free to react when attaching the crosslinking group. Although lysine residues are usually protected using the Fmoc group, sometimes the use of alternative protective groups is necessary, depending on the solubility and other chemical properties of the peptide. Subsequent deprotection strategies are dependent on the type of protecting group used.

In most cases, ligand probe synthesis can be achieved using solution-phase chemistry. However, if the peptide is very insoluble, dramatic loss of peptide may occur during purification following the conjugation and deprotection steps. In this instance, we advise a larger-scale on-bead synthetic approach that uses crude bead-attached peptide (as described in Alternative Protocol 1), which may also be purchased, if specifically requested, from some vendors (e.g. Genscript). Although on-bead synthesis of the ligand probe simplifies the conjugation and deprotection steps, the product is often of low purity which makes the purification more difficult. If this strategy is to be attempted, then the type of solid-phase resin must be known in order to properly release the probe ligand during the cleavage step.

An additional advantage of using crude bead-attached peptide is that it is generally less expensive compared to purified (> 95% purity) peptide, however the time and work needed to clean up the impurities from the ligand probe preparation may outweigh this benefit.

Choice of reporter molecule for detection of receptor-ligand complexes

Click chemistry allows for the addition of any number of azide-modified reporters, including affinity tags (e.g. biotin, FLAG) and fluorescent molecules (e.g. IR dyes, Texas Red, fluorescein). Selection of the reporter molecule depends on the resources available to the user. Major considerations for reporter selection include the availability of azide-conjugated reporter molecules and the detection methods available. Affinity tags detected by affinity blotting have the advantage of signal amplification due to enzyme-catalyzed enhancement of the chemiluminescence signal. Fluorescent molecules may not undergo the same degree of signal amplification (i.e. one molecule per receptor-ligand complex), but have the advantage of enabling detection of the fluorescence signal directly in the polyacrylamide which can afford faster detection. Subsequent affinity blotting of fluorescently-detected receptor-ligand complexes will still be necessary to detect the input receptor protein levels.

Safety considerations

Basic Protocol 1, Alternative Protocol 1, and Support Protocol 1 employ chemical synthesis and analytical steps that involve reagents and solvents that may be hazardous. Personal Protective Equipment (PPE) including safety glasses, gloves, laboratory coats, and closed-toed shoes must always be used when using these protocols. The Material Safety Data Sheets (MSDS) of all reagents should be consulted so that users are aware which reagents are toxic, flammable, corrosive, etc. Of special significance are steps that use piperidine for lysine protecting group deprotection (if applicable), Kaiser test reagents for on-resin reaction monitoring, trifluoro acetic acid for resin cleavage, and diethyl ether for ligand probe

precipitation. These steps must be performed in a fume hood, as their associated reagents are hazardous.

Protocol-specific instrumentation

Users must have access to a high-performance liquid chromatography (HPLC) instrument coupled to a mass spectrometer to identify and purify the ligand probe. The user must also be able to turn off UV lamps (normally used for detection of the compounds) to prevent premature crosslinking of the ligand probe during purification.

Microsome isolation and enrichment of plasma membrane fractions described in this unit require use of four different types of centrifuges: a microfuge for 1.5–2.0 ml tubes, a benchtop centrifuge for both 15 and 50 ml tubes, a refrigerated floor model centrifuge for 30 ml tubes at high (i.e. $15,000 \times g$) speeds, and an ultracentrifuge with a swinging bucket rotor capable of holding six 40 ml open-top tubes. This unit describes the use of the Beckman-Coulter Optima XE-90 ultracentrifuge with the SW 32-Ti rotor, but comparable instruments may also be appropriate.

An ultraviolet (UV) lamp with sufficient wattage and the required wavelength to activate the diazirine crosslinker is required. Since UV light can be dangerous to eyes and skin, appropriate PPE should be worn to avoid injury.

Sample treatments

For the plasma membrane-enriched microsomes (PMEMs) obtained at the end of Basic Protocol 2, we observed considerable variation in yield between different preparations. If multiple comparative treatments are necessary (as in the case of the example provided in Figure 9), we recommend splitting the sample at the beginning of Basic Protocol 3 into multiple aliquots to accommodate the multiple treatments. For the example result provided in Figure 9, we split one PMEM suspension sample into nine aliquots and could still readily detect the receptor proteins as well as the receptor-ligand complexes.

BASIC PROTOCOL 1

NOTE: Steps in this protocol can be performed at room temperature unless otherwise noted.

Generation of ligand probes

Ligand probes in this protocol include three functionalities: a trifluoromethyl-aryl diazirine for photo-crosslinking, an alkyne handle for ligation of biotin via copper-based click chemistry, and the peptide ligand. This protocol describes the N-hydroxy succinimide (NHS) activation of the carboxylic acid which facilitates ligation of the free amine on the N-terminus of the peptide ligand to the carboxylic acid (see Figure 2). If lysine residues are present, they are then deprotected. Crude ligand probes are HPLC purified to furnish the photo-crosslinker ligand probe.

Materials—NOTE: Reagent-grade chemicals can be used unless otherwise noted.

Trifluoromethyl-aryl diazirine alkyne acid (see above “Synthetic chemistry considerations”)

Dimethylformamide (DMF)

1-Hydroxybenzotriazole hydrate (HOBt)

N-hydroxy succinimide (NHS)

N-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide (EDC)

Argon or nitrogen gas (99.999% pure)

N,N-diisopropylethylamine (DIEA)

50% hexane/50% ethyl acetate solution

Peptide ligand; free or on-resin; lysine residues protected (see Strategic Planning for details)

Dimethylsulfoxide (DMSO)

1X PBS (see recipe)

8 ml glass reaction vials with caps

Pre-coated flexible TLC sheets, silica gel (Avantor Performance Materials, cat. no. 4449-02)

TLC solvent chamber

Glass capillary spotters

UV lamp with 254 nm emitted light (VWR, cat. no. 89131-492)

Rotary evaporator

Phase liquid chromatography Combiflash system (Teledyne, cat. no. 68-5230-021)

Lo-bind protein 1.5 ml tubes (Eppendorf, cat. no. 925000090)

Silica gel, 60-200 mesh (Avantor Performance Materials, cat. no. 3414-01)

Bench top or tray lyophilizer

NHS-activation of trifluoromethyl-aryl diazirine alkyne acid

1. Place 220 mg (0.67 mmol) of trifluoromethyl-aryl diazirine alkyne acid into a flame-dried 8 ml glass vial with magnetic stir-bar and dissolve in 4 ml dry DMF.

DMF can be dried by incubating DMF over flame-dried 3A molecular sieves overnight (20% w/v).

2. Add 111 mg (0.82 mmol) of HOBt to the reaction vessel.
3. Add 195 mg (1.69 mmol) of NHS to the reaction vessel.
4. Inject 0.145 ml (0.82 mmol) of EDC.

5. Run argon through reaction vessel for 15 minutes before injection of 0.291 ml (1.67 mmol) of DIEA.

If argon is not available, nitrogen gas can be used instead. Flushing with inert gasses displaces water vapor, and helps to obtain a higher yielding reaction.

6. Secure vial over stir plate and allow reaction to stir overnight at ambient temperature shielded from light.
7. Monitor reaction using thin layer chromatography with hexane/ethyl acetate solution as mobile phase solvent (R_f of 0.33 for the product, and 0.44 for the starting material).

TLC analysis (example in Figure 3) involves marking a line on the bottom of a TLC plate and using glass capillaries to spot the trifluoromethyl-aryl diazirine alkyne acid, the reaction, and both together. The spotted TLC plate is then set inside a chamber holding 10 ml of mobile phase such that the solvent travels up the TLC plate, which will move the compounds along the solid phase based on their polarity. When the solvent front is close to the end of the plate, remove the plate from the chamber, and mark the solvent front with a pencil. R_f values correspond to the relative distance that a spot moved based on the initial pencil mark and the solvent front. After the mobile phase dries, analyze the plate under UV irradiation. The aromatic scaffold will absorb the UV light, and appear as dark spots. The reaction is complete when the product spot is visible and no more trifluoromethyl-aryl diazirine alkyne acid starter remains. Refer to (Leonard, Lygo, & Procter, 2013) for more detailed information.

8. Upon completion of the reaction, remove the solvent via rotary evaporation, and isolate the desired product by normal phase liquid chromatography using silica gel. The hexane/ethyl acetate solution serves as the mobile phase.

Automated normal phase liquid chromatography can be performed on a Combiflash system (or equivalent) to facilitate product isolation.

9. Confirm product formation with MS.

A very dilute sample (up to 1 mg/10 ml) of isolated compound may be directly infused into the MS using electrospray positive ionization (ESI^+). The dominant product ion is the sodium adduct which has a mass-to-charge ratio of 448.1. Additionally, in source fragmentation of the product ion may result in a fragment ion with a mass-to-charge ratio of 420.1, corresponding to the loss of $N_2(g)$ (see example in Figure 4).

10. Remove the solvent via rotary evaporation and store dried NHS-ester probe (Figure 2) at $-20^\circ C$ in the dark.

Ligand probe conjugation

- 11 Weigh 1.7 mg of lyophilized peptide into a Lo-bind Eppendorf tube and dissolve in 0.25 ml DMSO. Add 0.125 ml of 1X PBS, and cool resulting peptide solution in an ice bath for 5 minutes.
- 12 Create a 7 mM stock solution of the NHS-ester probe by dissolving 11.5 mg in 3.86 ml DMSO.
- 13 Add 0.07 ml of 7 mM NHS-ester probe stock solution to peptide solution (from step 12), cover from light, and place on a rotary shaker or nutator and allow reaction to proceed for several hours to overnight.
- 14 Check the reaction with HPLC-MS (refer to Support Protocol 1) after three hours. If the reaction is <75% complete, allow the solution to shake overnight, and monitor again the following morning (see Figure 5 for an example of a nearly complete probe ligand conjugation reaction).
- 15 Once the reaction has proceeded to 75% according to HPLC-MS, lyophilize all solvent off to yield crude ligand probe (or protected ligand probe if lysine residues are present) (Figure 2).

Ligand probe deprotection

- 16 Purify the protected ligand probe using semi-preparative HPLC (refer to Support Protocol 1).

It is important to also recover the original unmodified peptide for future ligand probe conjugation attempts.
- 17 Subject lyophilized purified protected ligand probe to deprotection conditions based on the lysine residue protecting groups on the peptide.

As mentioned previously, deprotection conditions are not interchangeable, and the exact protocol will depend on the protecting group.

Depending on the deprotecting agent, this step should be completed in a fume hood to avoid inhalation of toxic substances.
- 18 Monitor the reaction using analytical HPLC-MS (refer to Support Protocol 1).
- 19 PLC purify crude deprotected product to obtain pure probe-ligand (refer to Support Protocol 1).e or less counted.anic solvent. Purify finished ligand probe with semi-preparative HPLC (refer to Support Protocol 1).

ALTERNATE PROTOCOL 1

Ligand probe synthesis on beads

In some cases, the free peptide may be difficult to handle due to solubility issues. In these cases, we have found it easier and more affordable to conduct conjugation and deprotection chemistry on the crude bead-bound peptide (Figure 6). This must be requested from the

peptide synthesis vendor. Loading of the peptide (mmol peptide/gram of beads), solid-phase synthesis residue (i.e. Rink amide resin), as well as the type of protecting groups present (if lysine residues are incorporated) must be known. However, because the peptide is crude, HPLC purification is significantly more difficult to obtain a pure probe ligand sample.

Materials—NOTE: Reagent-grade chemicals can be used unless otherwise noted.

Crude peptide-loaded beads from peptide vendor

N-methyl-2-pyrrolidone (NMP)

NHS-ester probe (see Basic Protocol 1)

N,N-diisopropylethylamine (DIEA)

Triethylamine (TEA)

Dichloromethane (DCM)

Kaiser test reagent A: 98% pyridine, 2% of 0.1% w/v KCN in distilled water

Kaiser test reagent B: 5% w/v ninhydrin in *n*-butanol

Kaiser test reagent C: 67% w/v phenol in *n*-butanol

Cleavage cocktail (made fresh once per daily use): Depends on solid-phase resin provided by peptide vendor

Dimethylsulfoxide (DMSO)

Dimethylformamide (DMF)

Diethyl ether

Argon gas

Glacial acetic acid

5 ml peptide reaction vessel with stopcock and cap (Peptides International, cat. no. SHG-20205-PI)

Glass Pasteur pipettes with rubber bulb

9 mm autosampler vials, screw thread caps, and inserts

Bench top or tray lyophilizer

15 ml conical bottom tube

Benchtop centrifuge and rotor for 15 ml tubes

4 ml glass vial

Ligand probe conjugation

1. To a dry 5 ml peptide reaction vessel, swell 200 mg of peptide-loaded beads in 1 ml dry NMP for 30 minutes. Preserve 0.05 ml of peptide resin slurry to monitor conjugation reaction.

Because chemical reactions on solid phase synthesis beads are diffusion limited, swelling is important to allow access of the NHS-ester probe to the bead-bound peptides. Use wide bore or cut 200 μ l pipette tips to allow transfer of peptide resin slurry.

2. Dissolve 25 mg (0.059 mmol) of NHS-ester probe into 3 ml dry NMP and add to the reaction vessel containing swollen peptide-loaded resin.
3. Add 0.005 ml of DIEA and 0.005 ml of TEA to the reaction.
4. Cap peptide reaction vessel and allow conjugation reaction to proceed on a rotary shaker or nutator.
5. Monitor the conjugation reaction every 2 hours using a Kaiser test until the reaction is >75% complete.

Kaiser tests expose beads to ninhydrin. If a free amine is present on the bead surface, then ninhydrin will form a blue compound and the beads will appear violet in color. Therefore, the less blue the beads are (compared to an initial aliquot of unconjugated beads), the further the reaction has proceeded since more free amines have reacted to the NHS-ester probe.

To conduct the Kaiser test, deposit a small aliquot of beads (~15 individual beads) into a test tube. Wash bead (3 $\times$ 1 ml) with DCM and dry. Using glass Pasteur pipettes, add 2–3 drops of Kaiser test reagent A, B, and C each. Heat the test tube for 5 min at 95°C which will cause beads to change color. Refer to (Wellings & Atherton, 1997) for more detailed information. Kaiser tests should be conducted in a fume hood to avoid inhaling heated Kaiser test reagents.

Conduct the Kaiser test on beads in the conjugation reaction and on beads from the original 0.05 ml peptide resin slurry. Beads from the original peptide resin slurry should turn dark blue due to the abundance of free amines. Beads from the conjugation reaction should be light blue or almost clear as the reaction nears completion.

6. Monitor the conjugation reaction using HPLC-MS. Cleave 25 beads with cleavage cocktail for 1 hour. Dry the supernatant down, dissolve residue in 0.05 ml of DMSO and transfer sample to an HPLC vial with insert. Inject 0.01 ml onto the HPLC-MS for analysis (refer to Support Protocol 1).

The recipe of the cleavage cocktail will depend on the type of resin and must be prepared fresh. If using trifluoro acetic acid, then conduct the cleavage steps in a fume hood to avoid inhaling the volatile acid.

Peptide sequence and lysine protecting groups must be known to observe the unconjugated peptide and the ligand probe (or protected ligand probe if lysine residues are present). After the peak area of the ligand probe or protected ligand probe is equal to, or greater than, the peak area of the unconjugated peptide, proceed to the next step.

7. Wash beads with DMF (4×5 ml) to remove unreacted NHS-ester probe, thereby ending the conjugation reaction.

Ligand probe deprotection

- 8 While still attached to the bead, swell the resin in NMP and deprotect all lysine residues according to their protecting groups.
- 9 Monitor the deprotection reaction with the Kaiser test (refer to above step 5).

As more lysine residues are deprotected, more free amines become available. Therefore, Kaiser tests will turn beads more blue as the deprotection reaction proceeds. As the beads turn dark blue to purple, the reaction should be nearing completion.
- 10 Monitor the deprotection reaction using HPLC-MS. Cleave 25 beads with cleavage cocktail for 1 hour. Dry the supernatant down, dissolve residue in 0.05 ml of DMSO, and transfer sample to an HPLC vial with insert. Inject 0.01 ml onto the HPLC-MS for analysis (refer to Support Protocol 1).

After the integrated area of the ligand probe is equal to, or greater than, the integrated area of the protected probe ligand, proceed to the next step.

Ligand probe cleavage

- 11 Wash beads by adding solvent, vortexing, and draining solvent via the stop cock at the bottom of the peptide reaction vessel.

Wash procedure includes the following: DMF (4×5 ml), DCM (4×5 ml), and diethyl ether (2×5 ml). Allow beads to air dry inside of the peptide reaction vessel.

This wash procedure is important to fully remove any conjugation and deprotection reagents/side products.
- 12 Cleave all beads by adding 1 ml of cleavage cocktail to the beads in the peptide reaction vessel and incubate for 2 hours.

Cleavage cocktail recipe and incubation time will vary depending on what amino acids are present, and on what solid phase resin is used. The cleavage cocktail should be made fresh on the day of cleavage. As mentioned above, if using trifluoro acetic acid, then conduct the cleavage steps in a fume hood to avoid inhaling the volatile acid.
- 13 Precipitate peptides by slowly draining cleavage cocktail into a 15 ml falcon tube containing 10 ml of cold diethyl ether. Centrifuge mixture at $1,000 \times g$ for 5 minutes to form a peptide pellet and remove solvent with a Pasteur pipette. Conserve the diethyl ether and store cleaved beads under argon.

This step must performed in a fume hood as ether is volatile and toxic.

Upon impact with cold diethyl ether, the draining cleavage cocktail will form a precipitate if its peptide concentration is high. If no precipitation occurs, transfer diethyl ether from falcon tube to a glass vial and remove solvent via rotary evaporation to yield a peptide residue, then proceed to the next step.

- 14 Dissolve the peptide pellet or residue with 2 ml glacial acetic acid, transfer to a 4 ml glass vial, and lyophilize. The peptide may now be purified with an HPLC instrument.

This step will exchange trifluoro acetate from TFA in the peptide salt for acetate.

- 15 Purify crude ligand probe product with HPLC (refer to Support Protocol 1).

SUPPORT PROTOCOL 1

HPLC-MS analysis and purification

Ligand probe synthesis requires the ability to monitor the peptide conjugation and deprotection reactions as well as the ability to isolate the desired products from reaction mixtures. This can be accomplished with an HPLC-MS, in which molecules are separated based on their polarity in a reverse-phase column, followed by ionization and compound analysis in the mass spectrometer. HPLC purification is very effective at separating compounds with even minor changes, and is thus well equipped to resolve peptides with and without the probe attached. On the other hand, predicted mass to charge ratios (m/z) of the expected products will easily allow for compound identification.

HPLC purification is achieved by repeatedly injecting small aliquots (up to 0.25 mg) of crude sample with the same HPLC method. For each run, a small amount of crude material is separated and, if a fraction collector is connected, deposited into flasks or vials. It is for this reason that method development is enormously important since any error at this stage is propagated throughout the entire purification. Refer to (Mant et al., 2007) for more detailed information.

Materials

Crude peptide

Glacial acetic acid (HPLC grade)

Acetonitrile (ACN) (HPLC grade)

9 mm autosampler vials, screw thread caps, and inserts

C12 Jupiter 250 × 10.0 mm semi-preparative HPLC column (Phenomenex, cat. no. 00G-4396-N0)

High performance liquid chromatography (HPLC) instrument

Mass spectrometer

HPLC fraction collector and glass vials or 125 ml flasks for fraction collection

Rotary evaporator

Bench top or tray lyophilizer

HPLC-MS analysis

1. Dilute a small amount of crude peptide in water (less than 0.3 mg/ml) and transfer to an HPLC vial with insert and cap.

If peptide does not dissolve, try dissolving the peptide in a final concentration of 50% ACN or 100 mM NaCl. Be sure there are no bubbles at the bottom on the HPLC vial insert.

2. Develop a general method to analyze peptide reactions.

HPLC methods require flow rate (dictated by the column), % organic phase, and injection volume. With increasing organic phase, peptides non-covalently bound to the hydrophobic column will be displaced, eluting as a chromatographic peak. Good separation is usually achieved by incorporating an equilibration step, a gradient with increasing ACN, a 100% ACN wash step, and then a rapid return to equilibration. If only the above semi-preparative column is used, then a good starting method for peptides would use a flow rate of 3.6 ml/min, 0.1% acetic acid in water for the aqueous phase (A), and 0.1 acetic acid in 100% ACN for the organic phase (B).

Begin with 10% B for 3 minutes, followed by a linear increase to 65% B over 25 minutes, a rapid increase to 100% B in 1 minute, holding 100% B for 4 minutes, decreasing to 10% B for 0.1 min and holding at 10% B for 2 minutes for a total of 35 minutes.

3. Inject 7 µl of crude peptide and analyze with mass spectrometry.

Ensure that electrospray positive ionization (ESI⁺) mode is being used for analysis on the mass spectrometer. Predict expected m/z values based on the peptide sequence, lysine protecting groups, and added probe. Constructing the fully modified peptide in chemical drawing programs allows for accurate m/z determination. Examine the chromatogram for species with multiple charges, especially for longer peptides as well as oxidized cysteine and methionine residues.

If HPLC-MS is being used to monitor a reaction, compare the peak areas of the desired product to peak areas of the unreacted species. As the reaction proceeds, product peak area should grow relative to the peak area of the unreacted species.

HPLC purification

4. Dissolve 2 mg of crude peptide product in 0.61 ml of water, deposit 0.3 ml into two HPLC vials with inserts and dilute the remaining 0.01 ml of peptide solution to 0.1 ml with water.

If peptides are insoluble in just water, add an organic solvent such as ACN or salt such as NaCl to fully dissolve. It is important that whatever solvent combination is used initially must also be used to dissolve the remainder of crude peptide during purification.

- 5 Construct an HPLC method capable of resolving all peaks of interest including ligand probe, unmodified peptides, oxidized variants, and lysine residue protected variants. See above for an example starting method.
- 6 Inject 25 μ l of diluted crude peptide mixture to verify the probe ligand by mass spectrometry in order to determine the fractions to collect.

First verify the retention times and identities for the ligand probe, the unmodified peptide, and oxidized variants of both forms. If available, attach the HPLC instrument to a fraction collector to collect peptide peaks based on the retention times observed in the mass spectrometer. Otherwise, collect peptide peaks manually in separate 125 ml flasks. Make sure that any UV lamps are turned off during fractionation.

- 7 Inject 25 μ l of the undiluted crude ligand probe solution and collect appropriate fractions containing the ligand probe. Repeat until the entirety of crude peptide solution has been purified.

At least 0.1 mg of peptide can be safely injected per run. Ensure that the HPLC injection loop is large enough to accommodate the injection volume. Additionally, collect fractions that span ~30 seconds before and after the peaks of interest.

- 8 Confirm that the collected fractions contain the purified ligand probe by injecting 25 μ l of each fraction onto the HPLC-MS.

If desired peptide is still contaminated, repeat purification with a modified HPLC method with a shallower linear % B gradient. If purification continues to be an issue, switch to a different column, or use a different organic solvent.

- 9 Remove solvent via rotatory evaporation, lyophilize, and reconstitute with water for a 1 mM solution of the ligand probe. Store in the dark at -20°C for up to 12 months. Lyophilized ligand probe can be stored long term at -80°C .

Final ligand probe may require organic solvents or higher salt content to fully dissolve. This solvent system must be noted and used for any future control experiments.

If desired, unreacted peptide fractions can be collected and used for future ligand probe synthesis. Prepare these fractions for long-term storage as described above.

BASIC PROTOCOL 2

Expression and purification of candidate receptor proteins

The purpose of this protocol is to overexpress and isolate affinity-tagged candidate receptor proteins in their native (i.e. plasma membrane) environment which can then be leveraged to evaluate ligand probe binding. The protocol first describes the process of generating large quantities of *Nicotiana benthamiana* leaf tissue transiently expressing affinity-tagged receptor proteins. Next, we detail the procedure to isolate microsomes and enrich the plasma membrane fraction using density gradient centrifugation. The final output is plasma membrane-enriched microsomes (PMEMs), which are used in Basic Protocol 3 to perform ligand binding and competition experiments (see experimental scheme depicted in Figure 7).

NOTE: Steps in this protocol can be performed at room temperature unless otherwise noted.

Materials

Agrobacterium tumefaciens with plasmids expressing affinity-tagged receptor proteins

Antibiotic solutions, as appropriate

LB (Luria-Bertani) solid media plates and liquid broth, both supplemented with antibiotics

Induction Media (see recipe)

200 μ M acetosyringone in 100% methanol (3',5'-dimethyl-4'-hydroxyacetophenone, Sigma-Aldrich cat. no. D134406)

Infiltration medium (see recipe)

Microsome extraction buffer (see recipe)

Microsome suspension buffer (see recipe)

Phase mixture (see recipe)

PEG (upper) and dextran (lower) phase solutions (see Support Protocol 2)

Cell spreaders

15 ml culture tubes

Benchtop centrifuge and rotor for 15 mL tubes

Spectrophotometer

1 ml needle-less syringes

Small gauge needles (optional)

4–6 week-old *Nicotiana benthamiana* plants

Foil, 50 ml conical bottom tubes, or other containers for storing frozen leaf tissue

Medium-sized mortars (11.5 cm) and pestles (18 cm)

Miracloth (EMD Millipore, cat. no. 475855)
Büchner two-piece filter funnel
500 ml filtering flask
High-speed centrifuge tubes, 30 ml capacity
Refrigerated floor centrifuge and appropriate rotor for holding 30 ml tubes
Ultracentrifuge (Beckman-Coulter Optima XE-90)
Ultracentrifuge rotor (Beckman-Coulter SW 32-Ti)
Ultracentrifuge tubes, 38.5 ml volume (Beckman-Coulter cat. no. 326823)
Immersion tip sonicator
50 ml conical centrifuge tubes

Preparation of *Agrobacterium tumefaciens* cultures

1. Prepare *Agrobacterium* strains containing plasmids necessary for transient expression of affinity-tagged receptor proteins of interest.

The antibiotics used in subsequent steps will depend on the antibiotic selection of the plasmid(s) as well as the *Agrobacterium* strain used.

More detailed information about *Agrobacterium*-mediated transient expression of recombinant proteins can be found in review articles such as (Canto, 2016; Curtis, 2016; Gelvin, 2003).
2. Streak the *Agrobacterium* strains onto LB solid media plates containing the appropriate antibiotics.

For each construct, patch a small amount of bacteria onto the plate and grow ~24 hours at 28°C. Then spread the culture over the entire plate using ~0.3 ml of LB broth and a cell spreader, and incubate overnight at 28°C.
3. Suspend the bacteria in induction media supplemented with acetosyringone (final concentration of 200 nM) and incubate for 3–5 hours at room temperature with shaking.

In general, one plate of bacteria can be suspended in 10 ml of induction media. Transfer bacteria from the plate to the induction media can be accomplished by suspending the bacteria in induction using a sterile cell spreader and then transferring the suspended bacteria to a 15 ml culture tube.
4. Wash *Agrobacterium* cultures and measure the bacterial concentrations.

Pellet the bacterial culture at maximum speed (i.e. $3,000 \times g$) for 5–7 minutes and wash the pellet twice with infiltration buffer. Resuspend the pellet in infiltration buffer and measure the optical density at 600 nm (i.e. OD₆₀₀) using a spectrophotometer.

5. Prepare infiltration solution using the suspended *Agrobacterium* culture diluted in infiltration media and supplemented with 200 nM acetosyringone.

Bacterial concentrations should be tested to determine the optimal concentration, and often ranges from OD₆₀₀ 0.05–0.5. Prepare enough solution to infiltrate the appropriate number of leaves needed. One leaf of fully-expanded *Nicotiana benthamiana* may require 1–5 ml of solution for complete infiltration, and may provide up to 2 grams of de-veined leaf material.

***Nicotiana benthamiana* leaf infiltration and tissue harvesting**

- 6 Syringe-infiltrate *N. benthamiana* leaves with the bacterial infiltration solution.

Use a needle-less 1 ml syringe and a small needle (if desired for poking holes to easily infiltrate the solution) to force the *Agrobacterium* infiltration solution into the apoplastic space of fully expanded leaves. It is often possible to infiltrate multiple (2–4) leaves per plant, though this depends on plant maturity and growth conditions.

- 7 After several days, harvest de-veined tissue, freeze in liquid nitrogen, and store at –80°C until needed.

Determine the optimum time for protein expression/accumulation, often 24–72 hours after infiltration. Harvest tissue by removing the most prominent veins carefully using a razor blade and freezing the remaining leaf tissue in liquid nitrogen. The tissue can be stored in foil, 50 ml conical bottom tubes, or other appropriate containers. It is helpful to weigh the tissue and aliquot in convenient quantities (such as 10 grams) for ease of use.

Additionally, it is recommended that small portions of the leaf tissue are tested to evaluate the starting quantities of receptor proteins before proceeding to the subsequent sections. This can be determined by affinity blotting and will add an additional two days to the time required, though tissue for several experiments can be evaluated at one time.

Protein extraction and isolation of microsomes

- 8 Grind 30 g of frozen *N. benthamiana* leaf tissue in a medium-sized mortar and pestle with liquid nitrogen. Add cold microsome extraction buffer and continue grinding.

Use 70–80 ml microsome extraction buffer for every 10 g of fresh weight tissue. As the amount of tissue needed more than readily fits in medium-sized mortars, we recommend processing tissue in 10 g aliquots. However, it might be possible to grind all 30 g of tissue in a very large (i.e. 21 cm) mortar.

It is important to keep the sample as cold as possible, though it is not necessary to perform the extraction in a cold room.

- 9 Filter extracted tissue sample through miracloth to remove excess debris.

Place a square of miracloth in the filter funnel and set funnel base in the top of the 500 ml filtering flask. If the seal between the funnel and flask is not tight, then wrap the base of the funnel with parafilm to achieve a tight fit. Attach tubing to the flask tabulation and a vacuum, and gently pull a vacuum. Transfer sample from mortar to funnel using a large volume pipette.

Once all liquid is removed from the debris, we recommend performing a back-extraction to harvest all available microsomes. Transfer the debris back to a mortar, add 20–30 ml of microsome extraction buffer, and grind. Use a new square of miracloth to filter the sample again.

It is important to keep the sample cold, so place the flask on ice during the filtering step.

- 10 Transfer cleared supernatant to pre-chilled 30 ml high-speed centrifuge tubes and centrifuge at $15,000 \times g$ for 25 minutes at 4°C.

Weigh tubes and adjust volume, if necessary, to achieve balanced tubes.

- 11 Transfer ~37 ml of cleared supernatant to six ultracentrifuge tubes and centrifuge at $100,000 \times g$ for 75 minutes at 4°C.

Depending on the ultracentrifuge, it may take up to 30 minutes to pre-chill the centrifuge, rotor, and tube holders.

It is **extremely important** that the tubes/samples are balanced for ultracentrifugation. It is recommended that all six samples/tubes are equal in weight (to within 0.01 g) so that there is not any ambiguity when loading the rotor. Do not place tubes on ice as the water on the outside of the tubes may alter the weight.

Isolation and enrichment of plasma membranes

- 12 Discard supernatant by inverting the six tubes with microsomal pellets.

See example in Figure 8a.

- 13 Add 8 ml of microsome suspension buffer **total** to the six tubes.

Add 0.5 ml to five of the pellets, and 5.5 ml to the last pellet.

- 14 Briefly sonicate with an immersion tip sonicator set to half power.

Place tubes in an ice bath to keep the samples cold during sonication. Try to minimize the amount of bubbles/froth produced.

Beginning with the one tube containing 5.5 ml of suspension buffer, sonicate in short bursts while keeping the sample cold. Once fully

suspended (no visible green pellet remaining), transfer the solution to one of the tubes containing 0.5 ml of suspension buffer. Sonicate as before, and transfer to another tube with 0.5 ml. Continue until all six pellets have been suspended. See example in Figure 8a.

- 15 Transfer 9 g (~9 ml) of suspended microsomal membranes to a 50 ml conical bottom tube containing 27 g of phase mixture. Thoroughly mix by inverting the tube 20 times.
- 16 Centrifuge in a benchtop centrifuge at 1,000 rpm ($200 \times g$) for 10 minutes at 4°C.
- 17 Transfer the clear upper layer to a new 50 ml conical bottom tube containing 7.5 ml of dextran (lower) phase solution. To the original 50 ml tube (containing the green lower layer) add 10 ml of PEG (upper) phase solution.
- 18 Mix both tubes by inverting 20 times and centrifuge at 1,000 rpm for 10 minutes at 4°C.
- 19 Transfer the clear upper layers from both tubes into a new 50 ml tube containing 7.5 ml of dextran (lower) phase solution.
- 20 Mix by inverting 20 times and centrifuge at 1,000 rpm for 10 minutes at 4°C.
- 21 Transfer clear upper layer to a new ultracentrifuge tube.
- 22 Centrifuge at 1,000 rpm for 5 minutes at 4°C and remove any contaminating lower phase solution. Fill to the top with microsome suspension buffer.

It is essential that there is no lower phase remaining, as this will prevent the formation of a pellet (see Figure 8b and c for example pellets).
- 23 Centrifuge at $100,000 \times g$ for 2 hours at 4°C.
- 24 Immediately proceed to ligand binding and photo-crosslinking (described in Basic Protocol 3).

SUPPORT PROTOCOL 2

Preparation of PEG (upper) and dextran (lower) phase solutions

The preparation of the phase solutions will likely take several days to complete, though the amounts of the solutions prepared should be sufficient for multiple experiments. It is important to be exact in the preparation of the phase solutions, otherwise the isolation of the plasma membranes from the other microsomal fractions will fail to occur. This particular phase solution recipe has been optimized for *N. benthamiana* leaf tissue, so if the user desires to utilize alternative plant tissues some optimization may be required. Several protocols proved useful in the development of our protocol and may provide useful information for developing an alternative protocol for other plant species (Alexandersson, Saalbach, Larsson, & Kjellbom, 2004; Kobayashi et al., 2007; Mitra, Clouse, & Goshe, 2009)

Materials

Polyethylene glycol (PEG), average molecular weight 3,350 (Sigma-Aldrich, cat. no. P4338)

Dextran from *Leuconostoc* spp. M_r 450,000-650,000 (Sigma-Aldrich, cat. no. 31392)

Sucrose

1 M potassium phosphate, pH 7.5

5 M NaCl

Vacuum-driven bottle top filters, 0.22 μ m

500 ml squibb pear-shaped separatory funnel with stopcock and stopper

Cold room or large refrigerator

Preparing the 40% PEG 3350 solution

1. Measure 200 g of PEG 3350.
2. Bring to 500 g with warm distilled water.
3. Heat and stir until fully dissolved.
4. Re-weigh solution and add additional water to a final weight of 500 g.
5. Filter-sterilize using a bottle top filter.
6. Store at 4°C for up to 12 months.

Preparing the 20% (w/w) dextran solution

- 7 Measure 50 g of dextran.

Wear a dust mask when handling the dextran as it can be an irritant if inhaled.
- 8 Bring to 250 g in hot distilled water.
- 9 Heat and stir until fully dissolved.

May take several hours to fully dissolve.
- 10 Re-weigh solution and add additional water to a final weight of 250 g.

This step is critical as significant amounts of water will be lost during the heating process.
- 11 Filter-sterile using a bottle top filter.

Due to the viscosity of the solution, it will take up to an hour to pass through the filter. Filtering while the solution is still warm will decrease the amount of time needed.
- 12 Store at 4°C for up to 12 months.

Preparing the PEG (upper) and dextran (lower) phase solutions

- 13 For 300 g of phase mixture, measure out the following and add to a bottle with a tight-fitting cap:
 - 45 g 40% (w/w) PEG 3350
 - 90 g 20% (w/w) dextran
 - 25.67 g sucrose
 - 3 ml 1 M potassium phosphate, pH 7.5
 - 1.74 ml 5 M NaCl
 - Bring to 300 g using warm sterile distilled water
- 14 Invert to dissolve and equilibrate to 4°C (several hours to overnight).
- 15 Mix well and transfer to a 500 ml separatory funnel. Allow to settle overnight at 4°C.
- 16 Collect the dextran (lower) and PEG (upper) phases separately. Solutions can be stored at 4°C for several months or longer at –20°C.

BASIC PROTOCOL 3

Formation and detection of receptor-ligand complexes

After successful isolation of plasma membrane-enriched microsomes, the next step is to bind and photo-crosslink the ligand probe to its receptor, further enrich the resulting receptor-ligand complex onto an affinity resin, and employ copper-based click chemistry to attach a reporter molecule azide to the alkyne handle included in the ligand probe. Elution from the affinity resin should yield an enriched receptor-ligand complex that can be western blotted and subsequently probed for the presence of ligand via the reporter molecule, and the presence of the receptor via its affinity tag. Alternatively, antibodies recognizing endogenous epitopes on the receptor protein may also be used. Detection of both receptor molecule and affinity tag in the same band, as well as absence of the reporter molecule in the appropriate negative controls, confirms direct receptor-ligand binding.

Materials

- Plasma membrane-enriched microsomes (PMEMs, from Basic Protocol 3)
- Plasma membrane (PM) binding buffer (see recipe)
- Affinity resin; specific type depends on the affinity tag present on the receptor protein
- 1X PBS buffer, pH 7.4 (see recipe)
- RIPA buffer (see recipe)
- Click chemistry master mix (see Support Protocol 3)
- Polyacrylamide gels; recommended 8% resolving gel
- 1X PAGE running buffer (see recipe)

Transfer buffer (see recipe)

TBS-T buffer (see recipe)

Blocking buffer (see recipe)

Primary and secondary antibodies

Affinity blot detection reagent

Stripping buffer (see recipe)

Sonicating water bath

Glass 9-well spot plate or other appropriate sample vessel

Blak-Ray B-100AP 100-watt lamp with 365 nm longwave bulb (UVP Ultraviolet Products, cat. no. 76005501)

Polyacrylamide gel electrophoresis (PAGE) apparatus

Transfer system

Receptor-ligand binding and crosslinking

1. Carefully remove supernatant and suspend PMEMs in an appropriate volume (~1–2 ml) of PM binding buffer.

Pellet is very loose, so remove supernatant carefully with a large volume pipette. **Do not** pour off the supernatant.

Depending on the number of treatments desired, suspend in 200 µl of buffer per treatment.
2. Keeping the sample cold, sonicate briefly using a sonicating water bath.

Sonicate in short bursts using lower power until PMEM pellet is fully suspended. Failure to completely suspend the pellet will result in unequal amounts of receptor proteins in the different treatment samples.
3. Pipette appropriate amounts of peptide ligand probe to different wells of a cold glass 9-well plate (as necessary), add 200 µl of PMEM suspension, and mix well.

Other vessels may be used to hold the samples during exposure to UV light. Appropriate vessels should be made of glass or other substances that will not react during UV treatment (i.e. not plastic) nor bind proteins, and should allow for maximal surface area while minimizing the liquid depth that the UV irradiation must penetrate. We found the 9-well plates to function well, and to allow for up to 9 simultaneous treatments.

This step needs to be performed in the dark to preserve the photo-crosslinking chemistry, and ideally in a cold room to keep the samples cold.
4. Incubate samples in the cold for 15 minutes in the dark.

5. Turn on UV lamp several (10–15) minutes in advance to allow time for the lamp to warm up.

Ultraviolet (UV) light can be dangerous to eyes and skin, so appropriate PPE should be worn to avoid injury.

6. Place UV light over the samples (working distance of 2.5 cm) and irradiate for 15 minutes.

Move the plate and lamp every 5 minutes to both mix samples and to keep the bench surface temperature low.

7. Turn off UV lamp and transfer each sample containing irradiated suspended membranes to a 1.5 ml tube.

After transferring each sample, rinse the glass with 0.8 ml PM binding buffer supplemented with detergents and transfer the buffer to the 1.5 ml tube. This rinse step helps to recover any residual receptor proteins that may have stuck to the glass surface.

Total volume of treated/crosslinked PMEM suspension plus detergent-containing binding buffer should be 1 ml.

Click chemistry-mediated reporter molecule addition to the ligand probe

- 8 Add an appropriate amount of prepared affinity resin (following the manufacturer's recommendations) to PMEMs suspended in detergent-containing binding buffer.

Prepare affinity resin by washing twice with cold PM binding buffer supplemented with detergents. Use wide bore or cut tips to facilitate pipetting the resin.

- 9 Incubate tubes overnight at 4°C on a rotary shaker or nutator.

- 10 The next day, wash the resin twice with cold 1X PBS buffer.

For this step and subsequent wash steps, use 250–750 µl of wash buffer for each wash, and centrifuge to pellet the resin between each wash step. Alternatively, if using magnetic resin, use a magnetized rack to aggregate the pellet.

- 11 Wash the resin three times with cold RIPA buffer.

- 12 Suspend the resin in 232.5 µl RIPA buffer and add 17.5 µl of click reaction master mix.

- 13 Incubate 2–6 hours at 4°C on a rotary shaker or nutator.

Reaction can be incubated as long as overnight at 4°C.

- 14 Wash the resin twice with cold 1X PBS buffer.

- 15 Wash the resin three times with cold RIPA buffer.

- 16** Upon removal of the final wash, suspend the affinity resin in 10 μ l of freshly prepared 3X Laemmli sample buffer.

Samples can be stored at -20°C for several weeks.

Detection of receptor-ligand complexes

- 17** Boil samples for 5 minutes at 95°C .

Some receptor proteins, especially multi-pass transmembrane proteins, may aggregate upon heating to 95°C , so instead heat to a temperature which is compatible with the receptor protein of interest. See Table 1 for further recommendations.

- 18** Perform polyacrylamide gel electrophoresis.

The use of low percentage gels are recommended to better resolve large receptor protein complexes.

- 19** Equilibrate gel in transfer buffer.

- 20** Transfer proteins from the gel to PVDF membrane.

Transfer time is 90 minutes at 100 V for wet transfer, or per the manufacturer's recommended conditions.

- 21** Block membrane in 5% blocking buffer for 1 hour at room temperature with shaking.

- 22** Move blot to TBS-T buffer with the primary antibody corresponding to the azide-reporter molecule added to the ligand (in above step 12).

Follow the manufacturer's recommendations for blocking and antibody conditions.

- 23** Incubate blot(s) overnight at 4°C with shaking.

- 24** Wash blot(s) with TBS-T buffer and incubate with second antibody (if necessary).

- 25** Perform protein visualization using an appropriate detection method.

- 26** After visualization of the receptor-ligand complexes is complete, strip the blot(s) using stripping buffer.

Handle stripping buffer in a fume hood, and conduct washes in a tight-sealing container to prevent inhalation of the β -mercaptoethanol.

Warm stripping buffer to 50°C and add to membrane in the container. Incubate for at least 15 minutes with gentle shaking. Dispose of the stripping buffer as appropriate, and rinse the membrane several times first with deionized water followed by several washes with TBS-T buffer. Continue washing until all traces of β -mercaptoethanol are removed, then proceed with blocking procedure.

Several stripping protocols are available. We observed the protocol described above to be efficient at removing the original antibodies while still preserving the integrity of the protein blot.

27 Probe blot using an antibody against the affinity tag present on the receptor.

This step is necessary to demonstrate the total amounts of the receptor protein present in the samples.

See example affinity blot in Figure 9, which was probed, stripped, and then re-probed to show the presence of both receptor-ligand complexes as well as total receptor protein.

SUPPORT PROTOCOL 3

Preparation of click chemistry master mix

For the copper-based click chemistry reaction to occur efficiently, it is essential that both the copper and reducing agent solutions are prepared fresh for each experiment. Additionally, although we utilized a biotin-labeled reporter, one advantage of this procedure is that the user can use their own preferred azide reporter molecule to conjugate to the ligand probe.

Materials

BTAA ligand (Chemical Synthesis & Biology Core at Albert Einstein College of Medicine, <https://einstein.ilabsolutions.com/>)

CuSO₄·5·H₂O

L-ascorbic acid sodium salt (Sigma-Aldrich, cat. no. A4034)

Azide-reporter molecule such as azide-PEG4-biotin conjugate (ChemPep Inc., cat. no. 271606)

1. Prepare 25 mM BTAA ligand in water, aliquot, and store at −20°C.
2. Prepare 5 mM azide-reporter molecule in water or per the manufacturer's recommendations, aliquot, and store at −20°C.
3. Prepare 12.5 mM cupric sulfate pentahydrate and 100 mM sodium ascorbate solutions immediately before use.
4. For each reaction, prepare the following:
 - 5 µl 25 mM BTAA ligand
 - 2.5 µL 12.5 mM CuSO₄·5·H₂O
 - 5 µl 100 mM L-ascorbic acid sodium salt
 - 5 µl 5 mM azide-reporter molecule

The solution will turn a faint blue color when the BTAA ligand and copper solutions are mixed, but then will return to a colorless

solution after the addition of the ascorbic acid. This color change can function as an indicator that the solutions are active.

5. For multiple reactions, prepare a master mix and aliquot into the appropriate number of tubes.

It is recommended to include extra for pipetting errors (i.e. 8.5X for 8 reactions).

6. Prepare fresh and keep on ice until use.

REAGENTS AND SOLUTIONS

Use deionized water in all recipes and protocol steps, unless otherwise noted.

AB salts, 20X

4 g NH_4Cl

1.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

0.6 g KCl

0.01 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$

0.4 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

Adjust pH to 7.0–7.5 using 1 M KOH

Bring to 200 ml with water

Autoclave and store at room temperature for at least 12 months

Induction media

4.88 g morpholino ethane sulfonic acid (MES)

Add ~400 ml water and adjust pH to 5.6 with NaOH

2.5 g dextrose

0.136 g NaH_2PO_4

Bring to 475 ml with water

Autoclave and store at room temperature for at least 12 months

Before use, add 25 ml of 20X AB Salts

Infiltration media

10 ml 1 M MgCl_2

20 ml 0.5 M MES, pH 5.7, filter-sterilized and stored at 4°C

Bring to 1 liter with sterile water

Store at room temperature for at least 12 months

Laemmli sample buffer, 3X

1.88 ml 1 M Tris-HCl, pH 6.8

1.5 ml 20% SDS

3 ml 100% glycerol

0.06 g bromophenol blue, or as much as needed to attain desired color

Bring to 8.5 ml with sterile water

Store at room temperature for at least 12 months

Before use, add β -mercaptoethanol (15% final concentration) to the buffer volume needed.

Prepare in a fume hood as the chemical is toxic and highly volatile. Do not store buffer with β -mercaptoethanol for more than a few days.

Microsome extraction buffer

12.5 ml 1 M MOPS-KOH, pH 7.5, filter-sterilized and stored at 4°C

62.5 ml 2 M D-sorbitol, filter-sterilized and stored at 4°C

1.25 ml 1 M DTT

2.5 ml 0.5 M EDTA

25 ml 10% polyvinylpyrrolidone (PVP; prepared fresh using sterile warm water to dissolve)

2.5 ml 0.1 M phenylmethylsulfonyl fluoride (PMSF) in 100% methanol, ethanol, or isopropanol

Bring to 250 ml with cold water; chill before use

Prepare fresh for each experiment

Preparation of the PMSF solution should be conducted in a fume hood as the chemical is toxic and can cause severe skin burns and eye damage.

Microsome suspension buffer

0.625 ml 1 M Tris-HCl, pH 7.5

2.14 g sucrose

0.25 ml 1 M Potassium phosphate, pH 7.5

0.144 ml 5 M NaCl

Bring to 25 ml with water; chill before use

Prepare fresh for each experiment

PAGE running buffer, 10X

30.0 g Tris base

144.0 g of glycine
10.0 g of SDS
Bring to 1 liter with water
Store at room temperature for at least 12 months

Phase mixture

10.8 g 20% (w/w) Dextran Mr 450,000–650,000, filter-sterilized and stored at 4°C
5.4 g 40% (w/w) PEG 3350, filter-sterilized and stored at 4°C
2.31 g sucrose
0.27 ml 1 M Potassium phosphate, pH 7.5
0.16 ml 5 M NaCl
Bring to 27 g using warm water to dissolve; prepare the day before needed and store at 4°C
Prepare fresh for each experiment
Weight components directly into a 50 ml conical centrifuge tube, except the sucrose which should be measured out first into a weigh boat to facilitate easy addition to the tube.

PBS buffer, pH 7.4, 10X

80 g NaCl
2 g KCl
14.4 g Na₂HPO₄
2.4 g KH₂PO₄
Adjust to pH 7.4 with HCl
Bring to 1 liter with distilled water
Store at room temperature for at least 12 months

Plasma membrane (PM) binding buffer

1 ml 1 M MES, pH 6.0, filter-sterilized and stored at 4°C
0.12 ml 1 M MgCl₂
0.08 ml 5 M NaCl
Bring to 40 ml with water
Prepare fresh for each experiment
Set aside 1–5 ml of buffer on ice for use in the binding step.

To the rest, add Triton X-100 (final concentration of 1%) and SDS (final concentration 0.01%). Mix well and chill on ice.

RIPA buffer

5 ml 10X PBS buffer, pH 7.4
0.5 ml 100% Triton X-100
2.5 ml 10% sodium deoxycholate
0.25 ml 20% SDS
Bring to 50 ml with water; chill before use
Store at 4°C up to 1 week

Stripping buffer

20 ml 10% SDS
12.5 ml 0.5 M Tris HCl, pH 6.8
Bring to 100 ml with water
Add 0.8 mL β -mercaptoethanol under the fume hood
Prepare fresh for each experiment

Preparation and use of the buffer should be conducted in a fume hood as the chemical is combustible and can cause severe skin and respiratory tract irritation.

TBS buffer, 10X

60.5 g Tris base
87.6 g NaCl
Adjust pH to 7.6 with 1 M HCl
Bring to 1 liter with water
Store at room temperature for at least 12 months

TBS-T blocking buffer

20 ml TBS-T buffer
1 g blocking reagent
Use immediately or store at 4°C for up to a few days

Blocking reagents can include non-fat dry skim milk, purified bovine serum albumin (BSA; Fisher Scientific, cat. no. BP1600), casein, or other commercially available products.

Shelf life can be extended to several weeks at 4°C with the addition of sodium azide to BSA-based blocking buffers. Storage of milk-based buffer is not recommended.

TBS-T buffer

100 ml 10X TBS

0.5 ml Tween₂₀

Bring to 1 liter with water

Store at room temperature for at least 6 months

Transfer buffer

2.93 g glycine

5.81 g Tris base

0.375 g SDS

200 ml methanol

Bring to 1 liter with water

Store at 4°C for at least one month

COMMENTARY

Background Information

Plant Receptor Biology—An area of increasing interest in the plant biology community is the identification of receptor proteins for ligand molecules important for immunity, growth, and developmental responses. In recent years, genetic and molecular biology approaches have revealed a number of putative immunity-related receptors (de Jonge et al., 2012; Jehle et al., 2013; Mosher et al., 2013; Pruitt et al., 2015; Ranf et al., 2015) but these proteins remain putative receptors due to the inability to demonstrate direct and specific interactions between the ligand and receptor. Without this important data, it can be difficult to discern a bona fide receptor from an essential but non-ligand-binding protein involved in perception or signaling. Our protocol enables the confirmation of direct receptor-ligand binding through photo-crosslinking, and detection of these complexes without the use of radiolabeled ligands. This method specifically describes the modification and use of peptide ligand probes but could be readily adapted, with minor modifications at the ligand probe synthesis protocol, for the study of other non-proteinaceous small molecule ligands [for example, (Park et al., 2012)].

Plant receptor-ligand interactions have primarily been demonstrated for immunity-related receptors using radiolabeled ligands in combination with chemical- or photo-crosslinking (Chinchilla, Bauer, Regenass, Boller, & Felix, 2006; Yamaguchi, Huffaker, Bryan, Tax, & Ryan, 2010; Zipfel et al., 2006). The method described in this unit offers the advantages of studying a covalently captured receptor-ligand complex but avoids the complications associated with using radioactive compounds for detection. Also, addition of the reporter

molecule to the ligand probe after photo-crosslinking via click chemistry circumvents the possibility of the reporter molecule interfering with receptor-ligand interactions. Another recent technique used to demonstrate ligand binding leveraged dye-labeled peptides (Butenko et al., 2014; Wang et al., 2016). For the dye-based technique, the receptor-ligand interaction is detected using a peptide ligand conjugated to a dye called acridinium ester. After binding of the modified ligand to resin-immobilized or insoluble receptor proteins, the interaction is quantified by measuring light emission from reaction of the dye with a citric acid solution.

Overall, the protocols presented in this unit provide an alternative approach that can be used to demonstrate direct interaction between receptor proteins and peptide ligands to discriminate between putative and bona fide receptor-ligand pairs. It will be up to the discretion of the user to select the method appropriate to their individual needs as well as availability of equipment, reagents, resources, and technical expertise. It is important to note that the procedures presented in this unit rely upon having candidate receptor proteins and ligands. These methods can be used to confirm interactions, but are not appropriate for the *de novo* determination of receptor proteins.

We have attempted to modify the methods described in this unit to enable capture and identification of unknown receptors, but to date these efforts have been unsuccessful. Briefly, we followed the procedure as described through the UV crosslinking step (Basic Protocol 3 step 7) with subsequent steps including the addition of an affinity tag via click chemistry for receptor purification/enrichment followed by identification using mass spectrometry. Unfortunately, we encountered several challenges using this modified protocol which we were unable to overcome. One major problem with this approach is that transmembrane proteins tend to be natively expressed at low levels, making their capture and identification using mass spectrometry challenging (Qi & Katagiri, 2009). We also found that the ligand-receptor binding buffer was incompatible with the click chemistry reaction conditions. A short investigation suggested that some components of the binding buffer (Basic Protocol 3 step 1), including $MgCl_2$, contributed to inhibition of the copper-based click reaction (Basic Protocol 3 step 12). We attempted to reconcile this incompatibility issue by way of buffer exchange, including the use of methanol:chloroform protein precipitation (Wessel & Flugge, 1984), but ultimately these efforts failed most likely due to limited receptor recovery post-precipitation or because of impractically large resuspension volumes. The methods described in this unit circumvent several of these issues by using affinity precipitation of the receptor protein (Basic Protocol 3 step 8) to function as both a buffer exchange and receptor enrichment step without needing protein precipitation; however, because this step requires an affinity or epitope tag on the receptor, it would not be possible for unknown receptors. Though we were thus far unsuccessful, we believe that future advances will make it possible to adapt the methods presented in this unit for *de novo* receptor protein identification.

Use of photo-crosslinking and click chemistry—The first requirement to confirm receptor-ligand binding with affinity blotting techniques is covalent bonding of the ligand to its receptor. This often entails a probe consisting of a photo-reactive group conjugated to a ligand. Upon activation by UV irradiation, the photo-reactive moiety forms a reactive

intermediate that quickly results in covalent bond formation between the probe and proximal targets in a process termed photoaffinity labeling (PAL). Such groups including diazirines, benzophenones, and aryl azides have been historically used to interrogate receptor-ligand interactions (Geurink, Florea, & Overkleeft, 2011). An example of bond insertion is given in Figure 10A for the case of diazirines, as used in this protocol.

The second requirement to confirm binding via affinity blotting is the ability to detect ligands and their receptors individually. While most receptors can easily be detected if overexpressed with affinity tags, ligand detection requires the presence of reporter molecules on the ligand itself (i.e. IR dyes, fluorescent dyes, affinity tags, radioactive groups). However, the added bulk of the attached reporter molecule may affect receptor-ligand binding. Therefore, a strategy is required to easily and selectively add reporter molecules to the ligand after binding. Click chemistry developed by Sharpless and Finn offers a set of rapid and selective ligation reactions to accomplish this task (Kolb, Finn, & Sharpless, 2001). The Copper Azide-Alkyne Cycloaddition reaction (CuAAC) is the quintessential click reaction and rapidly reacts azides with alkynes in the presence of Cu(I). Therefore, a probe bearing an alkyne can be selectively coupled to a reporter molecule containing the complementary azide (or vice versa.) This reaction can occur at room temperature under aqueous conditions, a reducing agent to reduce Cu(II) to Cu(I), and a Cu(I) chelator to stabilize the otherwise unstable Cu(I) catalyst (Figure 10B). The bioorthogonality of CuAAC makes this reaction an excellent choice to carry out the ligation of probe and reporter molecule in the presence of biological species.

Recently, a number of studies in plants have utilized click chemistry for detecting a variety of cellular processes including chemical inhibitor targets (Kaschani et al., 2009), cell wall lignification (Tobimatsu et al., 2014), cell cycle trafficking (Bourge, Fort, Soler, Satiat-Jeunemaitre, & Brown, 2015), as well as protein modifications such as farnesylation (Dutilleul et al., 2016), *N*-myristoylation and *S*-acylation (Boyle et al., 2016), and receptor-ligand binding (Hind et al., 2016). Given the relative ease of use and diversity of applications possible, adoption of click chemistry in plant research is likely to increase in the future.

Critical Parameters

1. It is imperative that the peptide ligand sequence is known, and all protecting groups are identified. Identification of HPLC peaks are determined by mass-to-charge ratios (m/z) which depend on peptide sequence. Failure to know this information may result in isolating peptides that do not have the probe attached, are already crosslinked to solvent or that have protecting groups still bound. These issues would all confound binding experiments.
2. Some peptides are notoriously sticky and are not easily dissolved. When preparing samples of these peptides for purification, the same dissolving solution must be used. Changing this solution between vials may slightly alter the retention time of peptides on the semi-preparative HPLC column.
3. In extreme cases of insoluble peptides, it is advisable to consider purchasing a dedicated C-12 semi-preparative column so that other purified peptides are not

contaminated with ligand probes. Specifically, we and others [e.g. (Mueller et al., 2012)] observed that the MAMP peptide flg22 absolutely must have its own column due to its insolubility, and should not be run on other columns used to prepare other peptides.

4. After purification of the ligand probe, it is important to confirm that the peptide retains biological activity. As the evaluation of activity may be unique for each receptor-ligand, the user will need to perform an activity assay appropriate for their own system. Similarly, adding an affinity tag the candidate receptor protein may interfere with binding and/or signaling functionality so it is important to access receptor function in an appropriate assay before proceeding with the binding assay described here.

Troubleshooting—For troubleshooting advice, see Table 1.

Anticipated Results

Ligand probe synthesis—NHS-activation of the diazirine scaffold should result in ~80% yield. Although ligand probe synthesis yield will vary depending on the peptide, 1 mg of peptide may yield 0.3–0.6 mg of ligand probe (with the remainder of the peptide easily recoverable for additional attempts.) For a bead-based synthesis, 200 mg of beads with 0.023 mmol/g peptide loading should yield around 5.5 mg of ligand probe. This estimation is based on our optimized yields of 80% conjugation and 50% lysine deprotection, and does not account for any loss during HPLC purification.

Plasma membrane-enriched microsomes (PMEMs)—We have observed an approximate yield of 200 µg of proteins from PMEMs starting from 30 g of *N. benthamiana* leaf tissue. Sensitivity of the assay will depend greatly on the sensitivity of the immunological reagents used (epitope tag, antibodies, chemiluminescent substrate, etc.).

Time Considerations—The procedural flow chart (Figure 1) provides an overview of the approximate time each protocol will take.

For the ligand probe synthesis (Basic Protocol 1), it will take around one month to synthesize the diazirine scaffold, about one month to receive the peptide preparation from the vendor, and then an additional 1–2 weeks to conjugate and purify the ligand probe.

For the transient expression of receptor proteins in *Nicotiana benthamiana*, the procedure will take 5–7 days; however, this estimate does not include the time needed to identify and clone the candidate receptor gene(s), which may take several weeks. The 5–7 day period includes the three days needed for growing and infiltrating the bacterial cultures as well as the time needed for protein expression. In our experience, optimal protein expression occurs within 2–3 days but may occur earlier or later, depending on the protein of interest. We have found that co-expression with the viral suppressor of silencing p19 reduces this variability and increases overall protein yield. Since the harvested *N. benthamiana* leaf tissue can be stored for up to several years in an ultralow –80°C freezer, large amounts of protein needed

for several experiments can be expressed and tested all at one time, thereby reducing the time needed for subsequent experiments to less than one week.

Extraction and purification of PMEMs for one sample takes at minimum 6 hours, and combined with the binding and photo-crosslinking (which must occur immediately following PMEM enrichment), processing of one sample takes at least 7 hours. Extraction of a second sample can be started during the two-hour centrifugation (step 22 in Basic Protocol 2), and as a result of these staggered start times two samples can be processed (from extraction through photo-crosslinking) in 11 hours.

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Figure 1.

Flow chart of the overall experimental procedure as described in this unit.

Figure 2.

Ligand probe synthesis begins with carboxylic acid activation of the trifluoromethyl-aryl diazirine alkyne acid, followed by amide bond formation between the *N*-terminus of the peptide and the activated carboxylic acid in 1X PBS buffer and DMSO. If protected lysine residues are present within the peptide, they must then be deprotected in order to yield the crude ligand probe. Protecting groups (PG) are indicated in green.

Figure 3.

This example TLC plate depicts the distance traveled, measured by retention factor (Rf), by purified trifluoromethyl-aryl diazirine alkyne acid (Rf of 0.44) and its corresponding NHS-ester probe product (Rf of 0.33) in a chamber of 50% hexane/50% ethyl acetate. Circled spots are visible under UV light. Staining TLC plates may also help with product spot differentiation.

Figure 4. MS spectra of the isolated NHS-ester product show the sodium adduct and a fragmentation ion resulting from the loss of $N_2(g)$ in electrospray positive ionization (ESI⁺).

Figure 5.

This example chromatogram depicts resolution of peptide peaks in a crude ligand probe sample. Peptides conjugated to the probe will typically elute later on C-12 semi-preparative columns due to their increased hydrophobicity. If methionine residues are present, oxidized products may also be observed.

Figure 6.

Ligand probe synthesis conducted directly on solid-phase peptide synthesis beads. The protecting groups (PG) are indicated in green while the synthesis beads are represented in blue.

Figure 7.
Experimental scheme depicting the procedures described in Basic Protocols 2 and 3. Figure modified from (Hind et al., 2016).

Figure 8.

Example pellets and suspensions observed during plasma membrane enrichment using density-gradient purification of crude microsomes. **(A)** Microsomal pellet is suspended in microsome suspension buffer and sonicated until fully suspended. **(B, C)** Plasma membrane-enriched microsomal pellets obtained after ultracentrifugation. **B** depicts a clean, well-enriched pellet while **C** shows a ring-like pellet contaminated with dextran (lower) phase, as described in the troubleshooting guide (Table 1).

Figure 9.

This example affinity blot demonstrates results of the analysis of receptor-ligand complexes (FLS3-flgII-28 complexes probed for the presence of the biotin ligand reporter-tag, top panel) and the input receptor protein (FLS3 probed for the GFP tag, bottom panel). This data is similar to what was shown in Figure 4e from (Hind et al., 2016) but comes from a different replicate experiment.

Figure 10.

Overview of the photo-crosslinking and click chemistry reactions. **(A)** Photo-crosslinking diazirine reacting upon excitation by UV light at wavelength 360 nm. The reactive intermediate carbene will insert itself between proximal covalent bonds found in the receptor protein. **(B)** Copper-catalyzed Azide-Alkyne Cycloaddition (CuAAC) reaction. Azide groups (on the left) react with alkyne groups (in the middle) in the presence of Cu(I) to form a triazole (on the right).

Table 1**Troubleshooting Guide**

Problem	Possible Cause	Solution
Low on-resin peptide modification (either conjugated to diazirine, or after deprotection)	Beads failed to swell	Swell beads in NMP or DCM
No ligand probes are detected	Peptide sequence is long and/or has basic amino acid residues	Search m/z values that correspond to higher charges
No ligand probes obtained from collected HPLC purification fractions	Retention time for ligand probe shifted after the first run	Examine collected fractions before and after the peak of interest. For future runs, inject several methanol blanks before HPLC method development and do not turn off the HPLC pump before conducting purification
Peptide is insoluble	Peptide is aggregating and/or very hygroscopic	Dissolve in known % of organic solvent and/or higher salt content
HPLC pressure exceeded and run stops during purification	Peptide may have precipitated at the start of the run, causing excessive pressure	Wash semi-preparative column according to manufacturer's instructions. Inject less volume (15 µl) upon resuming purification. If issue persists, clean column again and inject even less volume
Cannot resolve HPLC peaks	Compounds have similar polarities	Lower separation gradient so that peak(s) of interest are at the center of the gradient. If this fails, switch organic solvent. If this fails, use a different column
Peptide is not deprotected	Wrong deprotection procedure	Confirm identity of lysine protecting group and use appropriate deprotection method
Molecular weight of ligand probe is 10 daltons less than expected	Diazirine is activated and crosslinking to water	Store future aliquots of ligand probe in the dark. Turn off UV lamp during future HPLC purification attempts
Low yields of protein	Low expression of receptor protein	Use a viral suppressor of silencing such as p19 (Lindbo, 2007) or HC-Pro (Ma et al., 2009) to improve protein yields Use alternative vectors with different promoters or tags, or synthesize a synthetic version of the receptor gene with optimized codon usage
No PMEM pellet	Incorrect phase solution mixtures	Prepare all phase solutions carefully by exactly weighing out all reagents (up to 0.1 g)
Diffuse ring-like PMEM pellet (example in Figure 8)	Contamination of dextran (lower) phase solution	More carefully remove PEG (upper) phase solution, leaving behind the interface region. Check for contaminating dextran (lower) phase solution at step 21 in Basic Protocol 2
No receptor-ligand complexes detected	Little or no crosslinking occurred	Use a stronger UV source Increase crosslinking incubation time Shorten the path length between the sample and the lamp
	Click chemistry reaction was inefficient	Increase/decrease copper concentration Use an alternative azide reporter molecule or chelator
	Protein aggregation	Some transmembrane proteins, especially multi-pass, can aggregate upon heating. Test for tolerance of lower temperatures (see McLane, Hatzidimitriou, Yuan, McCann, & Ricaurte, 2007 as an example)
	Little or no target protein is transferred	Test alternative transfer buffer conditions (Abeyrathne & Lam, 2007)