Coupling surface plasmon resonance to mass spectrometry to discover novel protein–protein interactions

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The elucidation of protein–protein interaction networks is a crucial task in the postgenomic era. In this protocol, we describe our approach to discover protein–protein interactions using the surface plasmon resonance technique coupled to mass spectrometry (MS). A peptide or a protein is immobilized on a sensor chip and then exposed to brain extracts injected through the surface of the chip by a microfluidic system. The interactions between the immobilized ligand and the extracts can be monitored in real time. Proteins interacting with the peptide/protein are recovered, trypsinated and identified using MS. The data obtained are searched against a sequence database using the Mascot 2.1 software. Control experiments using blank sensor chips and/or randomized peptides are carried out to exclude nonspecific interactors. The protocol can be carried out in <3 days. Other methods, such as yeast two-hybrid systems or pull-down approaches followed by MS, are widely used to screen protein–protein interactions. However, as the yeast two-hybrid system requires protein interactions in the nucleus of yeast, proteins that are abundant in other compartments may not be detected. Pull-down approaches based on immunoprecipitation can be used to study endogenous proteins but they require specific antibodies. The protocol presented here does not require the specific labeling or modification of proteins.

INTRODUCTION

Increasing knowledge about protein–protein interaction networks is important to better understand many cellular and physiological processes. Furthermore, many disease states, including Alzheimer's disease and Parkinson's disease, are characterized by abnormal protein aggregation; therefore, disruption of protein–protein interactions could represent a novel therapeutic strategy for such disease states[1–3]. The aim of the protocol presented here is to describe the methodology used to identify new protein-protein interactions that exist in complex extracts and small volumes. We have used the surface plasmon resonance (SPR) technique to detect proteins interacting with a peptide or a protein of interest immobilized on a gold sensor chip[4,5]. We were interested in the identification of protein–protein interactions explaining neurodegeneration in Parkinson's disease. FKBP-12 is increased in a common rodent model of Parkinson's disease[4]; it is a neuroimmunophilin that acts as a receptor for the immunosuppressant drug, FK506, that is known to reverse neurodegeneration. We immobilized the entire FKBP-12 protein according to this protocol in order to determine novel interacting proteins that may provide further insight into the role of FKBP-12 in neurodegeneration. We have also used this protocol to identify proteins that interact with the voltage-gated sodium channel type X α-subunit (Nav1.8), a protein known to interact with the protein p11 (ref. 6), which we have shown previously to be dysregulated in depression-like states[7]. Caveolin-1 (Cav-1) was retrieved in recovery experiments with brain extracts injected into an immobilized peptide corresponding to the amino acid numbers 85–103 of Nav1.8 (Nav1.8 (85–103)). As Cav-1's scaffolding properties have not been fully explored, we immobilized the Cav-1 scaffolding domain (amino acids from 81 to 100 or Cav-1 (81–100)) onto the sensor chip and used brain extracts to show that this region is, indeed, the domain of Cav-1 interacting with Nav1.8 (ref. 5). Thus, the coupling of SPR and mass spectrometry (MS) can identify novel protein interactions that may further enhance our knowledge of disease states.

SPR

SPR is an optical technique used for characterizing interactions between macromolecules. It is based on a phenomenon occurring when monochromatic *p*-polarized light is reflected onto a gold-coated interface between two media[8,9]. The intensity of the reflected light is reduced at a specific incident angle (SPR angle). The SPR angle depends on the refractive index of the solution near the surface of the sensor chip, and is measured with an optical detection unit (**Fig. 1a**). Injection of macromolecules close to this surface modifies the refractive index and therefore alters the SPR angle[10]. For example, when proteins interact with an immobilized ligand on the surface of the sensor chip, the SPR angle is shifted, producing a signal measured in resonance units (RU) (**Fig. 1b**)[9,10].

Biacore AB launched the first commercial SPR biosensor in 1990. Currently, Biacore offers several models of SPR biosensors. Several other SPR biosensor systems have been developed by companies such as Texas Instruments, Affinity Sensors, IBIS Technologies, Quantech and BioTul AG. Classical sensor systems are based on the traditional glass prism-coupled SPR configuration[11]; however, silicon prisms or optical fibers have recently been developed[12]. The development of multi-analyte detection SPR will be an important step toward high-throughput screening. In SPR microscopy, an SPR image is obtained by measuring the reflected light intensity by a photo-array detector. Several methods, including microfluidic channel stamps, micro-contact printing, UV desorption, photo-oxidation, photolysis and electro-copolymerization, are used to pattern this SPR image (for review, see ref. 12).

SPR offers several positive aspects, such as versatility and ease of use. The protein–protein interactions are detected in real time and the amount of interacting proteins, rates of association and dissociation are measured with high precision[8]. No labeling or modification of the proteins is required[13]. Furthermore, SPR allows the analysis of interactions with a wide range of molecular weights, affinities and binding rates. However, measuring binding

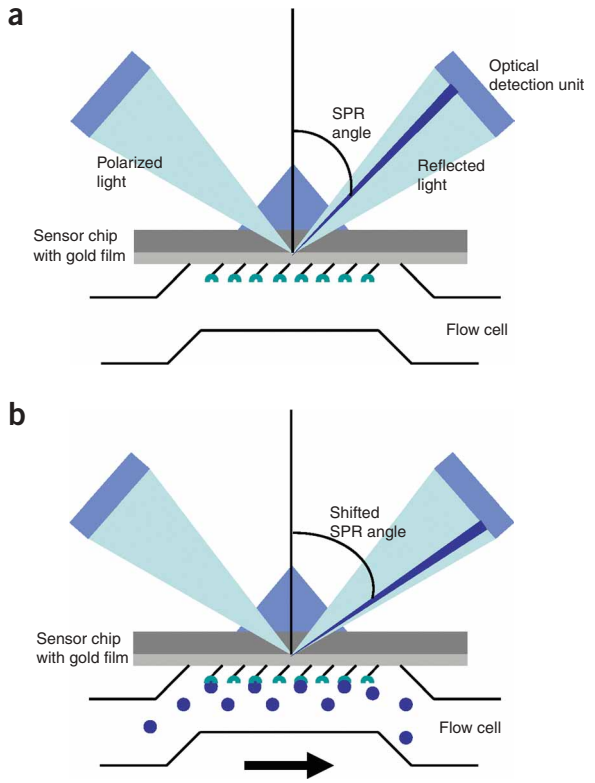


Figure 1 | The principle of surface plasmon resonance (SPR). (**a**) Polarized light is applied to the surface of the sensor chip and is reflected. The intensity of the reflected light is reduced at a certain incident angle, the SPR angle. (**b**) Interacting substances near the surface of the sensor chip increase the refractive index, which alters the SPR angle. The optical detection unit detects position changes of the intensity dips in the wedge of the reflected light corresponding to the SPR angle. The signals produced are measured in resonance units (RU).

interactions at low affinities may require careful optimization and proper controls, as specific binding may be small compared with nonspecific binding to the sensor chip or instrument[8]. Binding affinities can also be measured over a range of ionic strengths to describe the role of electrostatics in the interaction[14]. For example, the difference of the ionic strengths of protein–DNA interactions discriminates specific and nonspecific binding[15]. Moreover, the thermodynamic parameters of an interaction can be assessed by measuring the affinity and the binding rates at different temperatures[8]. SPR can also be used to study the conformational changes induced by the binding of a ligand to its receptor. For example, SPR analysis has shown that transforming growth factor-α induces a conformational change of the epidermal growth factor receptor resulting in its dimerization[16]. Sensor chips specifically designed to mimic the membrane surface allow the study of membrane interactions and can be a powerful tool for the characterization of membrane-integrated G-protein-coupled receptors[8]. More recently, SPR was used to implement cell-based assays to characterize endogenous G-protein-coupled receptors in adherent cells[17].

MS

MS is a crucial analytical technique used to identify and characterize proteins, and it has been shown to broadly affect biology and medicine[18]. Proteins analyzed by MS have to be ionized in the gas phase. The ions are separated according to their mass-to-charge ratio (m/z) and the relative abundance of the resolved ions is measured. Electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are the two primary methods for ionization, and they enable the mass determination of biomolecules and allow protein identification and quantification. In the MALDI method, a laser light is pulsed onto the sample molecules resulting in the sample ionization[19]. In the ESI method, electrical energy ionizes the analytes and transfers the ions from solution into the gaseous phase. ESI is easily coupled to liquid-based separation techniques such as capillary liquid chromatography (LC)[20]. The sequences of the peptides are obtained after their fragmentation by tandem MS (MS/MS). One peptide species out of a mixture is selected in the first mass analyzer and is then dissociated by collision with an inert gas. The fragments produced are separated in the second part of the tandem mass spectrometer, producing the MS/MS spectrum. The tandem MS spectra are matched against protein sequence databases using search engines such as X!Tandem or Mascot. The identity of the peptides is verified and hence the proteins making up the purified protein population can be established[21]. The significance of the results is determined by probability-based scoring. This calculates whether the probability that the observed matches between the data obtained and the peptides in the database is a chance event. The probabilities are converted into scores, and a high score implicates that the results are significant[22].

SPR/MS

Recent developments of both SPR and MS have allowed a better integration of these technologies, providing a powerful and sensitive tool dedicated to the identification and characterization of protein–protein interactions[13]. The combination of the two techniques offers very high sensitivity, which allows the use of very small amounts of extracts. Initial studies combined SPR with MS protein analyses directly on the sensor chip surface[23,24]. This approach allows a simple SPR/MS analysis and high sensitivity. However, SPR analysis can only be carried out once with the same sensor chip[25]. More recently, we and others have used target proteins eluted from the sensor surface for further MS analysis[4,5,25]. Nevertheless, this approach does not allow for the high-throughput analysis of various ligands of interest from a single SPR sensor chip. However, this is also true for yeast two-hybrid systems and pull-down MS approaches, in which individual ligands are screened for interactions.

In this protocol, a protein (FKBP-12) or a peptide Cav-1 (81–100) or Nav1.8 (85–103) is immobilized on a sensor chip. Brain extracts from mouse or rat are injected through the chip into the fluidic system. Noninteracting proteins from the brain extracts are then washed away, and proteins that have bound to the immobilized ligand are recovered by injection of the elution buffer. These interacting proteins are then recovered and digested into peptides that are separated and analyzed with a nano-LC LTQ MS/MS system. For identification of the interacting partners, the data are searched against protein sequence databases using Mascot 2.1 (**Fig. 2**). When RU measurements before recovery experiments and after elution are stable, there is no sign of protein accumulation. Thus, the sensor chip with immobilized peptides can then be used for several recovery experiments. Moreover, carboxymethylated sensor chips can be reused after immobilization experiments using a regeneration protocol[26].

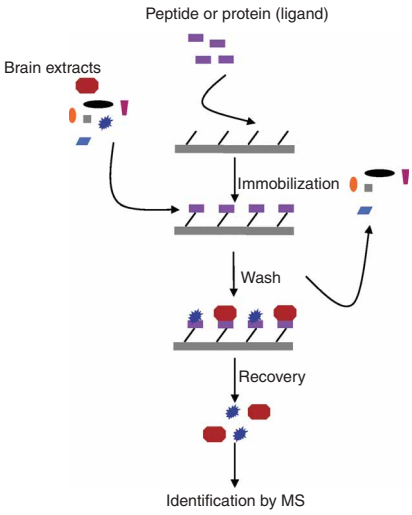


Figure 2 | Illustration of the presented protocol. The ligand is immobilized on a gold-coated sensor chip. Brain extracts from mouse are injected through this chip. After removing nonspecific interactions, proteins from brain extracts that have bound to the immobilized peptide are then eluted and identified using mass spectrometry (modified and reproduced with permission from ref. 5).

Applications

Elucidation of protein–protein interactions is one of the most obvious applications for SPR coupled to MS; however, it can also be used for other purposes. Indeed, this technique is relevant to screen for other types of interactions, such as compounds with specific receptors, transcription factors with specific DNA regions[27], biomarkers for certain diseases binding to specific proteins[26], inhibitors and substrates binding to specific enzymes[28] or proteins with posttranslational modifications or mutations that can influence their interaction capacity[27].

Limitations

Although the SPR/MS technique has many advantages, it should be emphasized that its high sensitivity increases the demand for optimization and controlled conditions[29]. Moreover, the amount of proteins that can bind to the sensor chip is limited. To increase the amount of bound protein, several cycles of binding and elution must be carried out. It should be noted that this increases the risk of accumulating nonspecific interacting proteins. Furthermore, this protocol is expensive in terms of purchasing the instruments as well as the maintenance.

Experimental design

The protocol presented here uses the Biacore 3000 for the SPR analyses. To obtain reproducible results, the conditions in the experiments require to be controlled. The different parameters that can be optimized are

- the operations and commands on the instrument;
- the sensor chip docking;
- the type of the sensor chip;
- the conditions used for immobilizations;
- the conditions used for recovery experiments;
- the gradient length and wash time of the LC method;
- the fine tuning of the electrospray conditions;
- the MS method setup; and
- the controls.

Control of the Biacore instrument. Operations of the Biacore instrument are controlled through informatics programs written by the user as text files in the Biacore Method Definition Language (MDL), which uses specific commands to control the different steps in the experiment. The user writes the programs according to the manuals provided by the supplier of the equipment. Examples of the programs used in this protocol are given in **Supplementary Figures 1–3** online. The commands are written in capital letters and are described in **Boxes 1–3**. Operations can also be carried out by an application wizard that guides the user through the experiment. This software tool is preprogrammed so that conditions of experimentation are predefined, and every step is automatically controlled. However, additional injections and washes can be added to the program if required.

BOX 1 | COMMANDS USED IN THE BIACORE MDL WHEN IMMOBILIZING PEPTIDES IN THE IFC

When immobilizing peptides in the IFC, the MDL is used to define each step. Below is the explanation of the different commands and their sequence used in the program.

- **FLOW 10.** Sets the flow to 10 µl min^{-1}.
- **FLOWPATH 1, 2, 3, 4.** Uses all four flow cells.
- **DILUTE 'position EDC' ' position NHS position' 'empty vial' 50.** Mixes equal volumes of EDC and NHS in the empty vial. 50 refers to the final percentage of the EDC in the mixture. The final volume of the mixture is fixed to 200 µl.
- **QUICKINJECT 'position empty vial' 70.** Activates the surface of the sensor by injecting 70 µl of the EDC-NHS mixture.
- Set report point as baselines 10 s before the injection of the mixture containing EDC and NHS. This is set by the user in the method program.
- **INJECT 'position peptide' 100.** Injects 100 µl of the peptide.
- Set report points as baselines 10 s before the injection of the peptide. This is set by the user in the method program.
- **QUICKINJECT 'position ethanolamine' 70.** Inactivates the surface of the sensor chip with the immobilized peptides by injecting 70 µl of 1 M ethanolamine.
- Set report points 10 s before the injection of the ethanolamine. This is set by the user in the method program.
- **FLOW 10.** Sets the flow to 10 µl min^{-1}.
- **WAIT 30.** 30 s break in the operation.
- **10 × QUICKINJECT 'position 1% (vol/vol) acetic acid' 5.** Injects 10 pulses of 5 µl of 1% (vol/vol) acetic acid.
- Set report points 10 s before the first injection of 1% (vol/vol) acetic acid. This is set by the user in the method program.
- **EXTRACLEAN.** Washes the IFC channels.
- Set report points 120 s after the last injection of 1% (vol/vol) acetic acid. This is set by the user in the method program.

BOX 2 | COMMANDS USED IN THE BIACORE METHOD DEFINITION LANGUAGE (MDL) WHEN CARRYING OUT RECOVERY EXPERIMENTS IN THE IFC

When carrying out recovery experiments in the IFC the MDL is used to define each step. Below is the explanation of the different commands used in the program.

- **FLOW 10**. Sets the flow to 10 µl min^{-1}.
- **MS_INJECT 'position running buffer' '%sample' 40 10**. Injects 10 µl of the brain extract, 10 mM OGP and 40 µl of running buffer.
- Set reports point as baselines 10 s before MS_INJECT. This is set by the user in the method program.
- **MS_WASH 'position 50 mM NaOH' 'position 2% acetic acid 50 mM OGP' 'position running buffer'**. Washes the IFC but not the flow cells.
- Set reports point 5 s before MS_WASH.
- **MS_RECOVER 'position water' 'position running buffer' 'position recovery solution' 'time' '%RecPos' 'flow cells'**. Washes the flow cells with running buffer and deionized water, injects the recovery solution (0.25% (vol/vol) TFA), which remains in the flow cells for an optional amount of time between two air segments, and elutes the recovery solution and transports it to the recovery vial.
- Set report points as baselines 33 s after the MS_RECOVER. This is set by the user in the method program.
- **FLOW 1**. Sets the flow to 1 µl min^{-1}.
- Set reports points 5 s before the change in flow. This is set by the user in the method program.
- **MS_WASH 'position 50 mM NaOH' 'position 2% (vol/vol) acetic acid 50 mM OGP' 'position running buffer'**. Washes the IFC.

The experiments are carried out at a predetermined temperature of 23 °C and the solutions are placed in vials with a cap specialized for the Biacore instrument. The user designates the position of the vials in the program. Then, the machine pipettes, injects and mixes the appropriate solutions in an empty vial according to the program. The user defines report points at certain times where the amount of RU is measured and allows the user to follow the progress of the interactions that occur, thus monitoring the experiments in real time. The signal correlates with the amount of protein interacting near the surface (1,000 RU correspond to 1 ng of bound protein per mm^2). Results are presented as sensorgrams, which plots RU against time (**Figs. 3–5**). Baselines are determined before injection of the ligand during the immobilization step and before the injection of brain extracts during the recovery experiments. The difference in the RU measured after the wash of the sensor chip and the baseline shows the amount of peptide or protein effectively immobilized on the chip surface (**Fig. 3**). Similarly, the measurement of the RU after the injection of the brain extracts shows the amount of proteins interacting with the immobilized ligand, whereas RU calculated after the last wash gives the amount of protein actually recovered (**Fig. 4**; see ANTICIPATED RESULTS).

Docking of the sensor chip. The sensor chip can be docked in the instrument using the Integrated µ-Fluidic Cartridge (IFC) or docked in the surface prep unit (SPU). The IFC contains a series of channels and pneumatic valves and forms four flow cells when pressed against the sensor chip. Thus, it becomes possible to immobilize four different ligands or the same ligand at four different protein concentrations, which is useful for the proper control conditions and during the optimization of the experimental conditions. However, for the purpose of this protocol, the same ligand is immobilized and the same extracts are injected into the four flow cells, as the eluated interacting proteins are recovered in one single tube. The commands used for immobilizing the peptide when the chip is docked in the IFC are described in **Box 1**.

The SPU is an external sensor chip holder with shorter injection and recovery channels. It can be set in two positions: the SP1 and SP2 positions. The SP1 position keeps the four flow cell format as in the instrument. In the SP2 position, a larger area flow cell format is created, which allows a higher capacity of immobilization of ligand in order to maximize the amount of recovered analyte. Thus, the SPU should be used when optimal conditions for immobilization and recovery experiments are already known and when the maximum amount of recovered interacting proteins is desired. It is important to note that MDL methods cannot be used to carry out an immobilization on a chip docked in the SPU. For this purpose, an application wizard called 'immobilization in the Surface Prep' must be used.

BOX 3 | COMMANDS USED IN THE BIACORE METHOD DEFINITION LANGUAGE (MDL) WHEN CARRYING OUT RECOVERY EXPERIMENTS IN THE SPU UNIT

When carrying out recovery experiments in the surface user prep unit, the MDL is used to define each step. Below is the explanation of the different commands used in the program.

- **FLOW 1**. Sets the flow to 1 µl min^{-1}.
- **MSP_PRIME**. Runs the working tool MSP_PRIME.
- **MSP_INJECT '%sample' 40 10.** 40 µl of the protein solution, 50 mM OGP is injected with a flow of 10 µl min^{-1}.
- **TRANSFER 'position 2% (vol/vol) acetic acid 50 mM OGP' ' position empty glass vial' '60'.** Cleans the needle by transferring 60 µl of 2% (vol/vol) acetic acid, 50 mM OGP to the empty glass vial.
- **MSP_RECOVER 'position 50 mM ammonium bicarbonate' 'position recovery solution' 'time' '%RecPos'.** Empties the injection port and washes with 50 mM ammonium bicarbonate, flushes the flow cell with 50 mM ammonium bicarbonate, incubates the flow cell in the recovery solution (0.25% (vol/vol) TFA) for 90 s and removes the recovery solution from the flow cell and transfer it to the recovery vial.

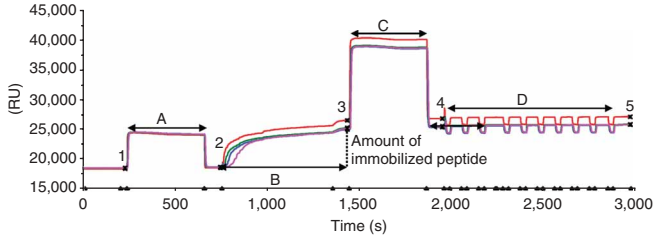


Figure 3 | Sensorgram of a representative immobilization. The four colored curves represent the four flow cells. The report points set during the experiments are denoted by numbers 1–5. Points 1 and 2 refer to the report points set 10 s before the injection of the EDC-NHS mixture and 10 s before the injection of the ligand, respectively. They are defined as baselines. A corresponds to the activation of the surface for amine coupling. Point 3 refers to the report points set 10 s before the injection of ethanolamine. B represents the time of injection of the peptide. Points 4 and 5 refer to report points set 10 s before the first injection of 1% (vol/vol) acetic acid and 120 s after the last injection of 1% (vol/vol) acetic acid, respectively. They indicate the amount of immobilized peptide after deactivation and washes with acetic acid when compared with the baselines. C represents the time for deactivation of the surface by ethanolamine. D represents 10 washes with acetic acid. All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

Sensor chip chemistry. Several types of sensor chips are suitable for different kinds of applications and immobilization approaches. The carboxymethylated sensor chips with streptavidin are used to immobilize biotinylated ligands, whereas pre-immobilized NTA sensor chips are suitable for the immobilization of HIS-tagged proteins. Other chips have a surface with carboxymethylated dextran matrix, which can vary in length or in the degree of carboxylation (CM sensor chips). Sensor chips with a high degree of carboxymethylation and long dextran molecules (CM5) have high immobilization capacities. In our hands, they have been very useful for the SPR/MS approach.

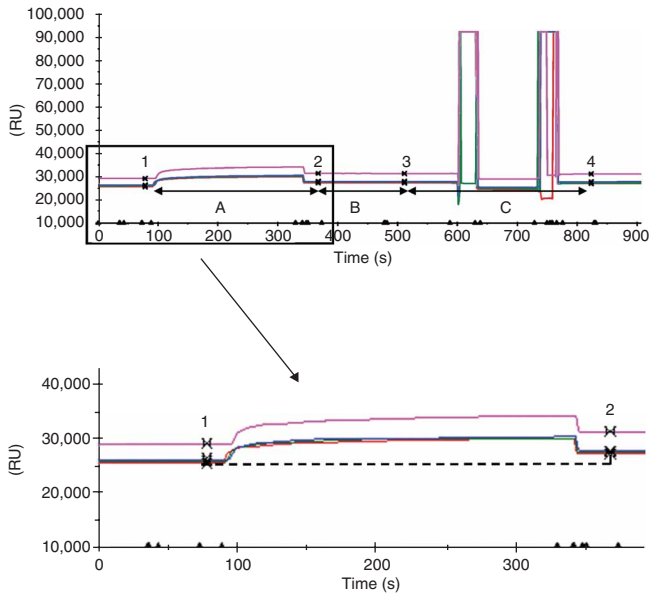
The ligand is covalently bound to the CM5 sensor chip by an amine coupling. It consists of the activation of the dextran matrix with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS), which leads to the formation of succinimide esters. These esters react with the amino groups of the ligand (peptide or protein) forming an amine bound between the sensor chip and the ligand. The remaining active esters are then deactivated by reaction with ethanolamine. Moreover, the ligand immobilized on the sensor chip is then washed with 10 pulses of 1% (vol/vol) acetic acid, which removes any electrostatically bound ligand.

Figure 4 | Sensorgram of a representative cycle of a recovery experiment. The four colored curves represent the four flow cells. The report points set during the experiments are denoted by numbers 1–4. Point 1 refers to the report points set 10 s before the injection of the brain extracts. They are defined as baselines. Point 2 refers to the report points set 5 s before washing the IFC after the injection of the brain extracts. They show an increase in the RU and the amount of bound proteins to the immobilized ligand. A corresponds to the injection of the brain extracts. Point 3 refers to the report points set during the time when the flow cells are washed with water and the running buffer. B represents the MS_Wash. Point 4 refers to the report points set 33 s after the injection of recovery solution and they show the amount of recovered proteins. C corresponds to the MS_Recovery. All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

Immobilization buffers. A suitable immobilization buffer is required to attach the ligand to the sensor chip. In fact, the peptide or the protein must be attracted to the surface by electrostatic forces in order to bind covalently to the matrix of the sensor chip. As the carboxymethylated dextran matrix carries a net negative charge above pH 3.5, the ligand should be positively charged. This occurs when the ligand is dissolved in a buffer with a pH lower than its isoelectric point. Consequently, the peptide or protein used as a ligand should be diluted in a buffer with a pH between 3.5 and its isoelectric point, which can be calculated using programs available on the internet. Buffers containing primary amines should be avoided, as amines can compete with the ligand for attaching to the carboxymethylated dextran matrix on the sensor chip by an amine bond. It should be noted that high ionic strength decreases the electrostatic interaction between the ligand and the dextran matrix. When optimizing this protocol, several different immobilization buffers such as Tris-HCl (50 mM; pH 4–6) or HBS-EP (50 mM) have been tested to dilute the ligand. Acetic acid (10 mM) and sodium acetate (10 mM) result in better and more reproducible immobilizations.

When optimizing the immobilization conditions, it should be considered that the immobilization levels will be higher if the ligand is a large molecule than if it is a small peptide. The contact between the injected ligand and the sensor chip is regulated by its concentration and the flow rate of the injection. The concentration of the ligand should be optimized according to its ability to interact with the activated sensor chip, which can be monitored by measuring the RU. Indeed, the RU measured when the ligand is injected through the activated chip surface correlates with the amount of ligand interacting with this surface. In this protocol, the peptides or the protein are diluted at a concentration of 100 μg ml^{-1}. The flow rates of injections can be slowed down to increase the contact time with the surface, thus increasing the binding of the ligand to the chip. In our experiments, a flow rate of 10 μl min^{-1} results in successful immobilizations[5].

HBS-EP is used as a running buffer during immobilizations. The running buffer circulates through the circuitry and avoids the accumulation of proteins on the walls of the fluidic system. This buffer is recommended by the supplier of the equipment; therefore, we have not tested any other type of solutions.



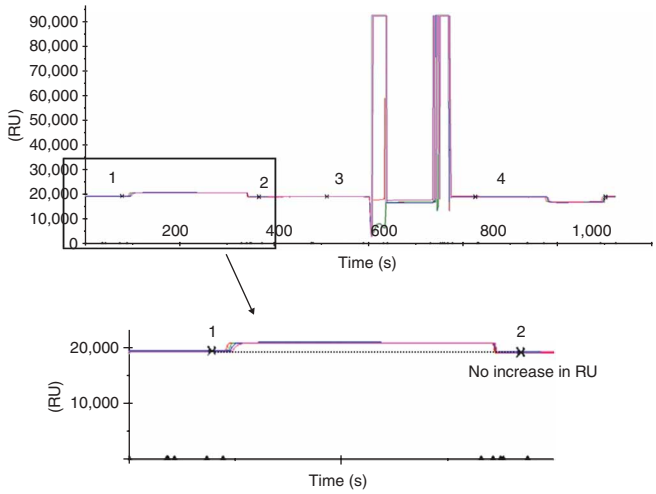


Figure 5 | Sensorgram of a representative cycle of a recovery experiment on a blank sensor chip. The four colored curves represent the four flow cells. The report points set during the experiments are denoted by numbers 1–4. Point 1 refers to the report points set 10 s before the injection of the brain extracts. They are defined as baselines. Point 2 refers to the report points set 5 s before washing the IFC after the injection of the brain extracts. There is no increase in the RU compared with the baseline showing no interaction between the brain extract proteins and the sensor chip. A corresponds to the injection of the brain extracts. Point 3 refers to the report points set during the time when the flow cells are washed with water and the running buffer. B represents the MS_Wash. Point 4 refers to the report points set 33 s after the injection of recovery solution and they show the amount of recovered proteins that will be considered as nonspecific interactors if retrieved in recovery experiments with an immobilized ligand. C corresponds to the MS_Recovery. All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

Interacting protein solution. The interacting protein solution can consist of complex tissue, in our case, brain extract from rodents (diluted at 50 μg ml^{-1}). It should be noted that the use of different types of extracts corresponding to subcellular compartments, such as synaptosomes or lipid rafts, can increase the concentration of specific interacting proteins. In this protocol, brain extracts are diluted in TBS buffer, which is compatible with MS experiments. During the recovery experiments, HBS-N is used as a running buffer. It has the same composition as HBS-EP buffer except that it does not contain surfactant P20 and EDTA. The latter two components need to be avoided, as they are not compatible with downstream MS experiments. Nonspecific hydrophobic interaction between the brain extracts and the immobilized peptide or protein can be reduced by adding specific detergents to the running buffer and the brain extracts[30]. We have used 50 mM octyl-β-D-glucopyranoside (OGP, 50 mM) as a detergent, as it is suitable for subsequent MS experiments. The brain extract solution is injected at a flow rate of 10 μl min^{-1}.

Recovery experiments. Recovery experiments are composed of several repetitive cycles of interaction between the brain extracts and the immobilized ligand followed by washing and elution of bound proteins (DEFINE LOOP in the commands). This multicycle approach is very useful to increase the amount of recovered proteins. However, too many cycles can lead to the accumulation of nonspecific interacting proteins.

The conditions of recovery experiments can be different if they are carried out in the IFC (option A) or in the SPU (option B). The MDL commands required for carrying out recovery experiments in the IFC and SPU are described in **Boxes 2** and **3**, respectively. The increased surface in the SPU induces a higher probability for nonspecific interactions between the brain extract solution and the immobilized ligand or the sensor chip surface. Thus, recovered experiments in the IFC are composed of 12 cycles, whereas recovery experiments in the SPU are composed of only 8 cycles. For the same reason, the concentration of OGP is higher in option B (50 mM) than in option A (10 mM). Moreover, the injection and recovery channels are shorter in the SPU than in the IFC, and the running buffer does not flush the system as effectively. Thus, we use ammonium bicarbonate (NH_4HCO_3) as an MS-compatible buffer in recovery experiments carried out in the SPU.

Elution buffers. The characteristics of the elution buffer depend on the type of bond between the ligand and the interacting proteins. Acids (formic acid, HCl, 10 mM glycine–HCl and H_3PO_4), bases (10 mM HEPES-NaOH, 100 mM NaOH and 10 mM glycine–NaOH), 25–50% (vol/vol) ethylene glycol, 10 M NaCl, 2–4 M $MgCl_2$ or 6 M guanidine chloride are alternative buffers that can be used (http://www.biacore.com/lifesciences/index.html). Usually, the stronger the interaction of the ligand with the protein solution, the stronger the acid, base or a higher concentration of salts should be used. Our experience shows that 0.25% (vol/vol) trifluoroacetic acid (TFA) successfully disrupts the bonds between the brain proteins and the immobilized ligand. The recovered protein mixture is then trypsinated either overnight at room temperature (19–22 °C) or for 1 h at 37 °C and then analyzed by LC-MS/MS. All buffers must be compatible with LC-MS/MS[27].

LC column considerations. The length of the gradient and the wash time of the LC column should be adapted to the nature of the sample. A complex sample, such as the recovery solution obtained after elution of the brain extract-interacting proteins, needs a longer gradient or pre-fractionation steps than a simple solution to obtain the best possible separation of the analytes. Furthermore, the presence of OGP (50 mM) used in the interacting protein solution, when eluted in the recovery experiments, complicates the LC separation, as it tends to stick to the columns. As OGP elutes at high concentrations of acetonitrile (ACN), extended column wash time and extra wash steps with 95% ACN between runs might be required to obtain a satisfactory separation. We have found that a 40-min gradient from 3% (vol/vol) solvent B to 65% (vol/vol) solvent B (see REAGENT SETUP) followed by a wash phase of 20 min with 95% (vol/vol) ACN results in the satisfactory separation of tryptic peptides from the recovered proteins[4,5].

Electrospray considerations. To obtain high sensitivity of the MS analysis, the distance between the spray needle tip and the mass spectrometer inlet, as well as the ionization spray voltage, should be optimized. The electrospray conditions are adjusted for each experimental setup. In this protocol, the voltage is set to 1.6 kV when Cav-1 (81–100) or Nav1.8 (85–103) peptides are immobilized on the chip in the SPR experiments and 1.9 kV when it is the FKBP-12 protein.

MS method setup. The correct protein identification by MS is critical for the successful application of SPR coupled to MS. Using an MS/MS method setup gives not only peptide mass data but also the amino-acid sequence information, making protein

identification more confident. The MS method is carried out in a data-dependent manner where highly abundant peptides that have already been analyzed by MS/MS are subsequently included in an exclusion list enabling sequencing of peptides of lower abundance. In this protocol, mass spectra are recorded in a data-dependent manner described in the EQUIPMENT SETUP section for 40 min.

Mascot 2.1. Mascot 2.1 is the search engine used for the identification of proteins by searching MS data against primary sequence databases. The program calculates the probability that the matches between the data obtained and the peptides in the database happens by chance. It converts this probability into MASCOT scores. We consider a MASCOT score above 27 as significant. The search parameters are set as follows: trypsin as the digestive enzyme, no fixed modification, potential oxidation of methionine, error window of experimental peptide mass values as 1.5 Da, error window for MS/MS fragment ions as 0.7 Da, peptide charge as 2+ and 3+, two missed cleavages, data file as MASCOT generic, instrument as ESI-trap and report hits as auto.

Controls. Experimental conditions must be appropriately controlled. Accumulation of proteins in the microfluidic system can lead to recovery of nonspecific interactors. To exclude this type of false-positive response, we carry out recovery experiments on blank sensor chips on which no ligand was immobilized (**Fig. 5**). The proteins retrieved in these experiments are excluded from the list of interacting proteins obtained in the experiments using an immobilized peptide or protein. Our experience is that around 50% of the interactions are false positive. Another control consists of immobilizing a randomized peptide, e.g., an oligopeptide of Cav-1 (81–100), that is a peptide of exactly the same amino acid composition but in a different order. The interacting proteins retrieved in these experiments with a randomized peptide ligand are not considered as interacting proteins to Cav-1 (81–100).

Table 1 presents a summary of the important parameters and the options used in this protocol.

TABLE 1 | A summary of important parameters and the options used in this protocol.

Parameter	Used in this protocol
Sensor chip	CM 5
Coupling	Amine coupling
Ligand	Nav1.8 (85–103) and Cav-1 (81–100) (peptides)/FKBP12 (protein)
Immobilization buffer	10 mM acetic acid 10 mM sodium acetate
Protein solution	Brain extract from mouse and rat Prepared with consecutive centrifugation and dilution in TBS
Detergent to avoid unspecific hydrophobic interactions	50 mM OGP
Regeneration buffer	0.25% (vol/vol) trifluoric acid
Length of LC gradient	3–65% (vol/vol) of solvent B; 40 min
Wash phase	20 min
Ion spray voltage	1.6–1.9 kV
Database	Swiss-Prot (Sprot_48.8.fasta)

LC, liquid chromatography; OGP, octyl-β-D-glucopyranoside. All animal experiments are carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

MATERIALS

REAGENTS

- Cav-1 (81–100) and Nav1.8 (85–103) peptides (Bachem)
- FKBP-12 protein (Abnova)
- Brain extracts (50 μg ml^{-1} in Tris-buffered saline (TBS) and 50 mM OGP) are obtained as described earlier[5] ! **CAUTION** All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).
- HBS-EP buffer, pH 7.4 (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% (vol/vol) Surfactant P20) (Biacore, cat. no. BR-1001-88)
- Amine Coupling Kit (containing EDC, NHS and 1.0 M ethanolamine) (Biacore; cat. no. BR-1000-50) ! **CAUTION** EDC is harmful and an irritant; wear suitable gloves and eye/face protection. ▲ **CRITICAL** Use frozen aliquots of EDC and NHS, as their efficacy decreases over time.
- Sodium acetate (Sigma, cat. no. S2889-1KG) ! **CAUTION** Sodium acetate is an irritant; wear suitable gloves.
- Hydrochloric acid (HCl) (Sigma, cat. no. 258148-2.5L) ! **CAUTION** HCl is corrosive; wear suitable gloves and eye protection.
- Acetic acid (CH_3COOH) (Sigma, cat. no. A6283-100ML) ! **CAUTION** CH_3COOH is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).
- Trizma base ($C_4H_{11}NO_3$) (Sigma, cat. no. T1503-1KG) ! **CAUTION** $C_4H_{11}NO_3$ is an irritant; wear suitable gloves and avoid inhalation.
- Sodium hydroxide (NaOH) (Biacore, cat. no. BR-1003-58) ! **CAUTION** NaOH is corrosive; wear suitable gloves and eye protection.
- HBS-N buffer pH 7.4 (0.01 M HEPES, 0.15 M NaCl) (Biacore, cat. no. BR-1003-69)
- OGP (Sigma, cat. no. 75083-5G) ! **CAUTION** OGP is an irritant; wear suitable gloves and avoid inhalation.
- TFA (Pierce, cat. no. 28902) ! **CAUTION** TFA harmful and highly corrosive; wear suitable gloves and eye/face protection.
- Sodium chloride (NaCl) (Sigma, cat. no. S7653-1KG)
- Sequencing Grade Modified Trypsin (Promega, cat. no. V5111)
- NH_4HCO_3 (Sigma, cat. no. A6141 1KG) ! **CAUTION** NH_4HCO_3 is an irritant; wear suitable gloves and avoid inhalation.
- HPLC grade water (Millipore)
- HiPerSolv acetic acid for HPLC (BDH, cat. no. 153103D) ! **CAUTION** Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).
- LiChrosolv ACN (Merck, cat. no. 1.14291.2500) ! **CAUTION** ACN is toxic; wear gloves when handling.
- LiChroSolv methanol (MeOH) (Merck, cat. no. 1.06007.2500) ! **CAUTION** Toxic; handle with gloves and protective glassware in a fume hood.
- ReproSil-Pur C18-AQ, 3 μm (Dr. Maisch GmbH, cat. no. r13aq)

EQUIPMENT

- Branson sonifier 250 (Branson Ultrasonics, cat. no. 063-197)
- Optima TLX Centrifuge System (Beckman Coulter, cat. no. A20690)
- Biacore 3000 SPR Sensor with a control software version 4.0 (Biacore)
- Sensor Chip CM5 (research grade) (Biacore, cat. no BR-1000-14)
- Plastic vials, 7 mm (Biacore, GE Healthcare, cat. no. BR-1002-12)
- Glass vials, 16 mm (Biacore, GE Healthcare, cat. no. BR-1002-09)
- Protein LoBind tube (Eppendorf, cat. no. 022431081)
- Eppendorf SpeedVac Concentrator 5301 (Eppendorf, cat. no. 022820109)
- Glass vials with blue screw caps (Agilent, cat. no. 5182-0714 (glass vial); cat. no. 5182-0717 (cap))
- Polypropylene insert, 100 μl (Agilent, cat. no. 5182-0549)
- Ettan MDLC system (GE Healthcare)
- Unicorn 5.01 (GE Healthcare)
- C18 PepMap100 solid-phase extraction m-Precolumn cartridge (particle size 5 mm, pore size 100 Å, 300-mm inner diameter; Dionex, cat. no. 160454)
- Fused silica emitter tip (o.d. 375 μm, i.d. 75 μm, tip 6 μm, length 150 mm; Proxeon Biosystems, cat. no. ES445)
- High-Pressure Column Packer and Sample Loader operated at 50–60 bars (Proxeon Biosystems, cat. no. SP036)

- LTQ Linear Ion Trap (Thermo Scientific)
- Xcalibur 1.4 SR1 software (Thermo Scientific)
- Software tool for compiling data files into MASCOT generic files (in-house written script)
- Mascot software (Matrix Science)

REAGENT SETUP

Cav-1 (81–100) and Nav1.8 (85–103) peptides dissolved in the immobilization buffer to 100 μg ml^{-1} or FKBP-12 protein dissolved in the immobilization buffer to 100 μg ml^{-1}. Adjust to the pH, if necessary (see 'immobilization buffers' section, above); use immediately.

1% (vol/vol) acetic acid Dilute 100 μl of 99.8% acetic acid in 10 ml of deionized water. Store at 4 °C for 1 week. ! CAUTION Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).

Immobilization buffer 10 mM acetic acid or 10mM sodium acetate. Adjust to a pH between 3.5 and the iso-electric point of the peptide with 0.1 M acetic acid; use immediately. ! CAUTION Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).

HBS-N buffer, 10 mM OGP Add 292.4 mg OGP to 100 ml HBS-N buffer. Store at 4 °C for 1 week.

2% (vol/vol) acetic acid, 50 mM OGP Add 146.2 mg OGP to 10 ml of 2% acetic acid; use immediately. ! CAUTION Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol). OGP is an irritant; wear suitable gloves and avoid inhalation.

Protein solution, 50 mM OGP Add 8.8 mg of OGP to 25 μg of brain extract proteins diluted in 700 μl of TBS buffer; use immediately. ! CAUTION OGP is an irritant; wear suitable gloves and avoid inhalation.

Recovery solution 0.25% (vol/vol) TFA. Make aliquots and store at −20 °C for 6 months or use immediately. ! CAUTION TFA is harmful and highly corrosive; wear suitable gloves and eye/face protection.

50 mM NH_4HCO_3 Add 39.6 mg NH_4HCO_3 to 10 ml deionized water. Store at 4 °C for 1 week.

0.1 M NaOH Add 39.997 mg of NaOH to 10 ml deionized water. ! CAUTION NaOH is corrosive; wear suitable gloves and eye protection.

TBS buffer 50 mM Tris-HCl, 150 mM NaCl, adjust to pH at 7.4. Store at 4 °C for several weeks. ! CAUTION TBS buffer is an irritant; wear suitable gloves and avoid inhalation.

Trypsin solution Dissolve trypsin in 0.1 mM HCl to a concentration of 0.02 μg ml^{-1}, freeze in aliquots at −20 °C and store for 6 months to 1 year.

0.25% Acetic acid (vol/vol) Dilute 25 μl of 99.8% acetic acid in 10 ml of deionized water. Store at 4 °C for 1 week. ! CAUTION Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).

Solution A 0.25% (vol/vol) acetic acid in HPLC grade water; use immediately. ! CAUTION Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).

Solution B 84% (vol/vol) ACN and 0.25% (vol/vol) acetic acid in HPLC grade water; use immediately. ! CAUTION ACN is toxic and corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol). ▲ CRITICAL When possible, de-gas and filter all reagents with a 0.22-μm filter. It avoids the accumulation of molecules and the clogging of the microfluidic system.

EQUIPMENT SETUP

Biacore 3000 instrument The Biacore instrument is controlled by the corresponding software BIAevaluation version 4.1. The programs are written as text files in the Biacore MDL. The positions of the different vials are chosen when writing the program according to the manufacturer's instructions.

Immobilization of the ligand The fluidic system is washed with the running buffer (HBS-EP), and then the sensor chip is activated by the addition of an equal volume of EDC and NHS, prepared according to the manufacturer's instructions. The ligand is injected at a flow rate of 10 μl min^{-1} before the inactivation of the sensor chip by the addition of 70 μl of 1 M ethanolamine and the washing of the chip by 10 pulses of 5 μl of 1% (vol/vol) acetic acid.

Recovery experiments The fluidic system is washed with the running buffer (HBS-N, 10 mM OGP). The brain extracts are then injected into the immobilized ligand (peptides or protein) on the sensor chip with 10 mM OGP and 40 μl of the running buffer. The IFC is washed with 50 mM NaOH, 50 mM OGP and the running buffer. The flow cells are then washed with running buffer and deionized water. The recovery solution is injected and remains in the flow cells for an optional amount of time, separated by air segments. Finally, the recovery solution is eluted and transported to the recovery vial before a final wash of the IFC with 50 mM NaOH.

Nano-LC MS/MS system The Ettan MDLC is connected to the LTQ linear ion trap to set up the nano-LC MS/MS system. The flow rate of the sample loading pump is set to 10 μl min^{-1} and the split flow over the analytical column is 200 nl min^{-1} in 3% (vol/vol) of solvent B. The sample is loaded onto the C18 PepMap precolumn from 0 to 6 min in 3% (vol/vol) solvent B, and then separated on the in-house packed spray emitter (ReproSil-Pur C18-AQ material) during a 40-min gradient from 3% (vol/vol) solvent B to 65% (vol/vol) solvent B, followed by a wash phase of 20 min with 95% (vol/vol) ACN, 95% (vol/vol) solvent B and a re-equilibration of 20 min at 3% (vol/vol) solvent B. Ion spray voltage is adjusted to 1.6 or 1.9 kV and the source temperature is set to 200 °C. Mass spectra are recorded from 12 to 52 min in a data-dependent manner. Every full-scan MS spectrum is followed by a zoom scan and a tandem MS spectrum of the peak with the highest intensity. Every peak is allowed to be picked twice before it is included in an exclusion list for 150 s.

PROCEDURE

Immobilization of the peptide ● TIMING 1–3 h

1| The immobilization of the protein or the peptide is operated by the instrument, and can be carried out with option A (the sensor chip docked in the instrument) or option B (in the SPU).

(A) Immobilization in the IFC controlled by an MDL method

(i) Dock a CM5 sensor chip using the menu command: DOCK.

(ii) Use HBS-EP as the running buffer. Place the buffer bottle on the location for the running buffer in the instrument.

(iii) Pipette 200 μl of the prepared ligand into a plastic vial with a cap specialized for the Biacore instrument.

(iv) Pipette 200 μl of EDS, NHS and ethanolamine into three different plastic vials with a cap specialized for the Biacore instrument. Pipette 600 μl of 1% (vol/vol) acetic acid into an additional plastic vial with a cap specialized for the Biacore instrument.

! CAUTION EDC is harmful and an irritant; wear suitable gloves and eye/face protection. Acetic acid is corrosive; wear suitable gloves and handle it in a fume hood when >25% (vol/vol).

▲ CRITICAL STEP Use an immobilization buffer that is suitable for the peptide. The pH of the immobilization buffer must be between 3.5 and the iso-electric point of the peptide. Optimize the concentration of the diluted peptide to obtain adequate levels of immobilization. Use vials with caps to minimize the risk of contamination and evaporation of the reagents.

(v) Place the vials with the reagents and one empty vial in the chosen positions.

(vi) Define the racks, temperature and detection in the MAIN block. Use type A rack and position it in rack base 2. Set the temperature to 23 °C and detection of all four flow cells.

(vii) Include the working tools PRIME and FLUSH in the MAIN block and end it with APPEND STANDBY.

(viii) Open the MDL method for immobilizing the ligand (peptide or protein) on a CM5 sensor chip (**Supplementary Fig. 2**).

(ix) Click on Run, choose Run Method.

■ **PAUSE POINT** The sensor chip with the immobilized peptide can be left in the instrument for a couple of days if the running buffer is changed to HBS-N. The sensor chip, if separated from the protective plastic housing cassette, can also be stored in a falcon tube filled with HBS-N at 4 °C for 3 days (**Supplementary Fig. 1**).

? TROUBLESHOOTING

(B) Immobilization in the SPU controlled by an application wizard

(i) Dock the sensor chip in the SPU, which is set in the SP2 position.

(ii) Position the SPU in rack base 2 and rack type A in rack base 1.

▲ **CRITICAL STEP** A sensor chip has to be docked in the instrument, even if the SPU is used.

(iii) Run MSP_PRIME.

(iv) Choose Run: Application Wizard from the main menu.

(v) Choose Surface Preparation and click on start.

(vi) Choose Immobilization in Surface Prep. Click on next.

(vii) Choose Surface Prep type 2 as configuration, to immobilize the peptide in one large flow cell instead of four flow cells connected in a series. Click on next.

(viii) A dialog box will appear, displaying the three default injection types required for amine coupling: mixing and injecting EDC and NHS, injecting the peptide and injecting ethanolamine. Click on Add.

(ix) Choose Extra wash after injection with 50 mM NaOH and water. Click on Ok and Next.

(x) Pipette 63 µl of EDC, 56 µl of NHS and 92 µl of ethanolamine into three different plastic vials with a cap specialized for the Biacore instrument.

! CAUTION EDC is harmful and an irritant; wear suitable gloves and eye/face protection.

(xi) Pipette 885 µl of 0.1 M NaOH and 885 µl of deionized water into two additional plastic vials with a cap specialized for the Biacore instrument.

! CAUTION NaOH is corrosive; wear suitable gloves and eye protection.

(xii) Pipette 92 µl of prepared ligand into a plastic vial with a cap specialized for the Biacore instrument.

(xiii) Place the solutions prepared in Step 1B(x) in the positions displayed in the dialog box. Save the template, click on Start.

(xiv) Undock the sensor chip from the SPU when the immobilization is complete.

(xv) Dock it in the instrument and run the working tool PRIME with HBS-EP.

■ **PAUSE POINT** The sensor chip can be retained in the instrument for a couple of days if the running buffer is changed to HBS-N. The sensor chip, if separated from the protective plastic housing cassette, can also be stored in a falcon tube filled with HBS-N at 4 °C for 3 days.

Recovery of proteins bound to the immobilized peptide ● TIMING 4–5 h

2| The recovery experiment is operated by the instrument and is controlled by a written MDL method. It can be carried out with option A (the sensor chip docked in the instrument) or option B (the sensor chip docked in the SPU).

(A) Recovery experiment in the IFC controlled by an MDL method

(i) Dock the sensor chip containing the immobilized peptide in the instrument using the menu command: DOCK.

(ii) Use HBS-N, 10 mM OGP as the running buffer. Place the buffer bottle on the location for the running buffer in the instrument.

! CAUTION OGP is an irritant; wear suitable gloves and avoid inhalation.

▲ **CRITICAL STEP** The running buffer must be compatible with MS.

(iii) Run the working tool PRIME.

(iv) Position rack type A in rack base 2 and reagent rack in rack base R.

(v) Pipette 600 µl of brain extracts diluted in 50 mM OGP, 50 mM NaOH, 2% (vol/vol) acetic acid in 50 mM OGP and the recovery solution (0.25% (vol/vol) TFA) into separate plastic vials with caps.

All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

! CAUTION NaOH is corrosive; wear suitable gloves and eye protection. OGP is an irritant; wear suitable gloves and avoid inhalation. TFA is harmful and highly corrosive; wear suitable gloves and eye/face protection.

(vi) Pipette 5 ml of the running buffer and deionized water into 16-mm glass vials.

(vii) Place the vials in the chosen positions in rack type A and also an empty LoBind Eppendorf tube (recovery vial) in the recovery position in reagent rack.

(viii) Define the racks and flow cells in the MAIN block. Use type A rack, positioned in rack base 2, and reagent rack, positioned in rack base R. Set detection of all four flow cells.

(ix) Include the command LOOP with ORDER in the MAIN block to get a repetition of the following commands: FLUSH and APROG.

(x) Set the parameters required in the APROG to % sample, %RecPos and %Analyte Name, and define them 12 times in the DEFINE LOOP block as the position of the protein solution, the position of the recovery vial and the name of the protein solution, respectively.

(xi) End the MAIN block with APPEND STANDBY.
(xii) Open the MDL method for binding and recover brain extracts proteins against a ligand (peptide or protein) in the IFC (**Supplementary Fig. 2**).
(xiii) Click on Run, choose Run Method.

▲ CRITICAL STEP It is preferable to run the maintenance procedures DESORB and SUPERCLEAN on a regular basis to decrease the risk of carryover between recovery experiments. As SDS is used in these procedures, it needs to be washed with water or buffer to wash away SDS deposits; this detergent should be avoided in MS experiments.

■ PAUSE POINT After the recovery experiment, the sensor chip can be docked in the SPU overnight. However, it is preferable to undock the sensor chip and store it in HBS-N in a falcon tube at 4 °C for 3 days. It can be used for at least three recovery experiments (**Supplementary Fig. 2**).

? TROUBLESHOOTING

(B) Recovery experiment in the SPU controlled by an MDL method

(i) Dock the sensor chip containing the immobilized peptide in the SPU, which is set in the SP2 position.
(ii) Dock an unused sensor chip in the instrument.

▲ CRITICAL STEP A sensor chip has to be docked in the instrument even if the SPU is used.

(iii) Position the SPU in rack base 2, rack type A in rack base 1 and reagent rack in rack base R.
(iv) Use HBS-N as the running buffer. Place the running buffer bottle on the location for the running buffer in the instrument.

▲ CRITICAL STEP The running buffer must be compatible with MS.

(v) Pipette 600 µl of brain extract diluted in 50 mM OGP, 2% (vol/vol) acetic acid in 50 mM OGP and the recovery solution (0.25% (vol/vol) TFA) into separate plastic vials with caps.
All animal experiments were carried out according to the local ethical committee at the Karolinska Institute (Application N282/06).

! CAUTION OGP is an irritant; wear suitable gloves and avoid inhalation. TFA is harmful and highly corrosive; wear suitable gloves and eye/face protection.

(vi) Pipette 5 ml of 50 mM NH_4HCO_3 and water into two glass vials.

! CAUTION NH_4HCO_3 is an irritant; wear suitable gloves and avoid inhalation.

(vii) Place the vials in defined positions in rack type A, an empty LoBind Eppendorf tube (recovery vial) and an empty glass vial in defined positions in reagent rack.
(viii) Define the racks and flow cells in the MAIN block. Use type A rack, positioned in rack base 1, and reagent rack, positioned in rack base R. Set detection of all four flow cells.
(ix) Include the command LOOP with ORDER in the MAIN block to get a repetition of the APROG command.
(x) Set the parameters required in the APROG to %sample, %RecPos and %Analyte Name, and define them eight times in the DEFINE LOOP block as the position of the protein solution, the position of the recovery vial and the name of the protein solution, respectively.
(xi) End the MAIN block with APPEND STANDBY.
(xii) Use the MDL method for binding, and recover brain extract proteins against a ligand (peptide or protein) in the SPU (**Supplementary Fig. 3**).
(xiii) Click on Run, choose Run Method.

■ PAUSE POINT The flow cell and the injection port are filled with the running buffer after the MSP_RECOVER; therefore, the sensor chip can be docked in the SPU overnight. However, it is preferable to undock the sensor chip and store it in HBS-N in a falcon tube at 4 °C for 3 days. It can be used for at least three recovery experiments (**Supplementary Fig. 3**).

? TROUBLESHOOTING

Trypsination ● TIMING overnight

3| Add 5 µl of trypsin solution and 45 µl of 50 mM NH_4HCO_3 (pH 8) to the recovery vial containing the brain protein solution that is obtained from the recovery experiment.

4| Incubate overnight at room temperature.

■ PAUSE POINT The trypsinized protein solution can be stored at −20 °C for several weeks.

Nano-LC MS/MS analysis of the recovered proteins ● TIMING overnight

5| Lyophilize the trypsinated protein solution in a SpeedVac for 2 h.

▲ CRITICAL STEP Do not dry completely because this reduces the recovery of peptides.

6| Dissolve the peptide mixture in 8 µl of 0.25% (vol/vol) acetic acid and mix by pipetting up and down 5–10 times.

7| Transfer the peptide solution to the polypropylene inserts and place them in the glass vials in the autosampler of the Ettan MDLC.

8| Use Unicorn to control the Ettan MDLC. Start a run by specifying an injection volume of 5 µl and the pickup location of the vial in the autosampler. Run the method as indicated in the EQUIPMENT SETUP. The Ettan MDLC sends a start signal to the LTQ MS 12 min after injection.

9| Use the Xcalibur software to control the LTQ linear ion trap MS.

10| Configure the LTQ MS to start at the contact closure signal from the Ettan MDLC. Record mass spectra for 40 min. Full-scan MS spectra are recorded between *m/z* 300 and 2,000. One zoom scan spectrum and one MS/MS spectrum are recorded for the peak with the highest intensity in the preceding full-scan MS spectrum. Allow one peak to be picked twice before it is included in an exclusion list for 150 s. Place the autoproteolytic fragments of trypsin on the exclusion list.
▲ **CRITICAL STEP** The distance between the spray tip and the mass spectrometer and the ion spray voltage should be optimized to obtain a high sensitivity detection of peptides before analyzing important samples. The ion source of the LTQ MS should be cleaned and the LTQ MS should be calibrated on a regular basis to obtain spectra of high quality.
▲ **CRITICAL STEP** As OGP tends to stick to the column system and elute at a high percentage of ACN; extra washes of the columns with 95% (vol/vol) ACN can be included when running a batch of samples.
? TROUBLESHOOTING

Data processing ● TIMING 1–2 h
11| Convert the raw LTQ data into dta files with Xcalibur 1.4 SR1 and assemble them into combined MASCOT generic files (mgf).

12| Use MASCOT to search the mgf files against the Swiss-Prot database. Choose the taxonomy suitable for the analysis.

13| Upload the mgf file from the experiment and set the search parameters as explained in the EQUIPMENT SETUP. Consider hits that have a score higher than the cutoff score suggested by Mascot to be true peptide hits.
? TROUBLESHOOTING

● TIMING
Step 1, Immobilization of peptide: 1–3 h
Step 2, Recovery of proteins bound to the immobilized peptide: 4–5 h
Steps 3 and 4, Trypsination: overnight
Steps 5–10, MS identification of the recovered proteins: overnight
Steps 11–13, Data processing: 1–2 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A	No immobilization	Imperfect immobilization buffer Too low concentration of the immobilized peptide/protein	Optimize the pH and the salt content of the immobilization buffer Increase the concentration of the immobilized peptide/protein
2A	No binding of interacting partners	Time of contact between the interacting partners and the sensor chip surface is not sufficient	Decrease the flow rate when injecting the tissue extract
2B	No recovery of bound proteins	The elution buffer does not break the bonds between the interacting partners	Optimize the elution buffer
	Accumulation of proteins on the immobilized chip after recovery	The elution buffer does not break the bonds between immobilized ligand and interacting proteins	Optimize the elution buffer or increase the time of the wash at the end of the recovery experiments
10	Clogging of the analytical column	OGP accumulation on the analytical column	Wash the column system extensively with a high percentage of acetonitrile
13	No peptide matches	No trypsin digestion	Prepare fresh trypsin solution
	A large amount of unspecific binding	Contamination with nonrelevant proteins as keratin, tubulin, myelin basic protein, actin, hemoglobin, etc.	Wear protective clothes and/or increase the amount of washing of the microfluidic system of the instrument

ANTICIPATED RESULTS

As an example of results, we briefly present below published data from experiments that followed this protocol and where either a protein or a peptide were used as a immobilized ligand[4,5]. This protocol led to the identification of proteins with potential interactions with FKBP-12, a neuroimmunophilin that acts as a receptor for the immunosuppressant drug FK506 (ref. 4). We have also used the SPR technique coupled to MS to identify a novel protein interaction between Cav-1 and Nav1.8 (ref. 5).

Immobilization of the ligands

Figure 3 shows a representative sensorgram from an immobilization of Cav-1 (81–100). The four colored curves correspond to the four flow cells. In this protocol, the four flow cells are used for immobilizing the same ligand at the same concentration; consequently, they look similar. The report points are set to facilitate the interpretation of the sensorgram. They are denoted with numbers, each corresponding to four report points, one for each curve. Point 1 refers to the report points set 10 s before the injection of the EDC-NHS mixture, and they are defined as baselines. Point 2 refers to the report points set 10 s before the injection of the peptide, and they are also defined as baselines. They show the effect of activation of the sensor chips surface on the resonance signal. Point 3 refers to the report points set 10 s before the injection of ethanolamine; these are of interest as they indicate the amount of immobilized peptides by showing the increase in RU after the injection of the peptide (between points 2 and 3). This shows that the ligand bound effectively to the activated chip surface. The difference in RU between points 3 and 4 corresponds to a modification of the refractive index induced by the injection of different buffers. Furthermore, points 4 and 5 refer to report points set 10 s before the first injection of 1% (vol/vol) acetic acid and 120 s after the last injection of 1% (vol/vol) acetic acid, respectively. They indicate the amount of immobilized peptide after deactivation and washes with acetic acid.

Recovery experiments

The recovery experiments with the sensor chip docked in the instrument were carried out in triplicate on the same previously immobilized sensor chip. Each recovery experiment contains 12 cycles and each cycle contains three steps:

- interaction between the brain extract and ligand;
- washing of nonspecific interactors; and
- elution of bound-interacting proteins.

A representative sensorgram from one cycle in a recovery experiment is presented in **Figure 4**. Similar to the sensorgram in **Figure 3**, the four colored curves correspond to the four flow cells. They also look identical, as the same brain extracts are injected in all four flow cells where the same ligand was immobilized. The report points are denoted with numbers. Point 1 refers to the report points set 10 s before the injection of the brain extracts, and they are defined as baselines. Point 2 refers to the report points set 5 s before washing the IFC after the injection of brain extract. These report points show an increase in the RU and the amount of proteins bound to the immobilized ligand, thus indicating that the experiment was successful. Point 3 refers to the report points set during the time when the flow cells are washed with water and the running buffer. These report points show the amount of available proteins before exposing the surface to the recovery solution. Similar to **Figure 3**, the difference in RU between points 3 and 4 corresponds to a modification of the refractive index induced by the injection of different buffers. Finally, point 4 refers to the report points set 33 s after the injection of recovery solution and they show the amount of recovered proteins.

In order to distinguish between specific and nonspecific interactions, control recovery experiments have been carried out on blank sensor chips (**Fig. 5**). Proteins retrieved in these experiments were excluded from the analysis when recovered in the experiments with a peptide or a protein immobilized on the sensor chip. Moreover, to exclude nonspecific interactions with Cav-1, recovery experiments on a randomized peptide (Cav-1 RP) immobilized on the sensor chip have been carried out (data not shown). This Cav-1 RP has the same amino-acid content than Cav-1 (81–100), but in a different order.

MS analyses

The results from the MS analysis of the trypsinated protein solution obtained in the recovery experiments suggest a vast number of proteins interacting with FKBP-12, Nav1.8 or Cav-1. A mass spectrum is given as example in **Supplementary Figure 4**. It is assigned to a peptide derived from Cav-1 retrieved in recovery experiments when Nav1.8 (85–103) was immobilized on the sensor chip. It contains the tandem MS spectrum and the matched fragment ions (*b*, *y*-ions) in a table format. A protein is considered as a potential interactor if one of the following criteria is fulfilled:

- Mascot scores above a significant cutoff value suggested by the search engine;
- at least two tryptic peptides are detected; and
- peptide is retrieved in at least two recovery experiments carried out with the same immobilized ligand.

A representative set of proteins that we identified and met our criteria is listed in **Tables 3–5**.

Proteins identified in the peptide solution from blank chip recovery experiments and/or from recovery experiments with immobilized Cav-1 RP are excluded. In our experiments, proteins that bind to blank chips include keratin, myelin basic protein,

TABLE 3 | Proteins recovered from the experiments with mouse and rat brain extracts on immobilized FKBP-12 protein.

Species	Accession number	Name	Number of recovery experiments detected	MASCOT score	Number of identified peptides	Sequence coverage (%)
Mouse	O08709	1-cys peroxiredoxin protein	1	54	2	28
	P63101	14-3-3 zeta	1	75	2	15
	P07356	Annexin A2	1	60	2	22
	Q04447	Creatine kinase, brain	1	146	5	40
	P63017	Heat shock 70 protein	1	207	8	20
	P52480	M2-type pyruvate kinase	1	126	8	19
	P08551	Neurofilament triplet L protein	1	65	3	14
	P62631	Statin-like, Elongation factor 1-alpha 2	2	250	6	28
				40	1	5
Rat	P63102	14-3-3 zeta	2	90	2	24
				42	1	24
	P04764	Alpha-enolase	5	84	3	20
				79	2	15
				74	3	15
				68	2	12
				40	2	12
	P62630	Elongation factor 1-alpha 2	3	66	1	16
				56	1	10
				51	1	12
	P63018	Heat shock 70 protein	2	54	1	8
				33	1	4
	P11980	M2-type pyruvate kinase	3	57	4	9
				35	1	6
				32	1	16
	P35704	Peroxiredoxin-2	1	53	1	9

Accession number refers to the number used to archive the protein in the Swiss-Prot database. The sequence coverage is expressed as the number of amino acids spanned by the assigned peptides divided by the sequence length. All animal experiments are carried out according to the local ethical committee at the Karolinska Institute (Application N282/06). Reproduced with permission from ref. 4.

actin, tubulins, hemoglobin subunits, ribosomal proteins, histone proteins, alpha/beta-caseins and some microtubule-associated proteins.

The suggested interacting partners to FKBP-12 are listed in **Table 3**. Several proteins, including 14-3-3 zeta, heat shock 70 protein and M2-type pyruvate kinase, are retrieved in recovery experiments with both mouse and rat brain extracts showing a homology of the potential interactions between species.

The suggested specific interacting partners to Nav1.8 (85–103) are listed in **Table 4**. Cav-1 is retrieved in a significant manner in both species. The suggested specific interacting partners to Cav-1 (81–100) are listed in **Table 5**. Interestingly, Nav1.8

TABLE 4 | Proteins recovered from the experiments with mouse and rat brain extracts on immobilized Nav1.8 (85–103) peptide.

Species	Accession number	Name	Number of recovery experiments detected	MASCOT score	Number of identified peptides	Sequence coverage (%)
Mouse	P49817	Caveolin-1	1	66	1	8
	O08553	Dihydropyrimidinase-related protein 2	2	62	3	6
				58	3	6
Rat	P41350	Caveolin-1	4	64	2	12
				51	2	8
				33	2	8
				27	1	5
	P45592	Cofilin-1	2	32	1	7
				30	1	7
	P47942	Dihydropyrimidinase-related protein 2	3	40	2	4
				32	4	5
				27	5	7
	P47819	GFAP	2	42	2	3
				36	2	6

Accession numbers refer to the number used to archive the protein in the Swiss-Prot database. The sequence coverage is expressed as the number of amino acids spanned by the assigned peptides divided by the sequence length. All animal experiments are carried out according to the local ethical committee at the Karolinska Institute (Application N282/06). Reproduced with permission from ref. 5.

TABLE 5 | Recovered proteins from the experiments with mouse and rat brain extracts on immobilized Cav-1 (81–100) peptide.

Species	Accession number	Name	Number of recovery experiments detected	MASCOT score	Number of identified peptides	Sequence coverage (%)
Mouse	P49817	Caveolin-1	5	109	1	8
				60	1	8
				59	1	8
				55	1	8
				44	1	8
	P18160	Cofilin-1	3	111	3	21
				96	1	8
				59	1	8
	P17183	Gamma-enolase	3	111	2	5
				87	3	10
				74	2	4
	Q64018	Glycine receptor alpha-1 chain precursor	4	50	1	1
				38	3	5
				37	1	1
				35	1	1
	O55131	Septin-7	4	52	2	8
				43	3	9
				39	4	11
				38	5	12
	P54227	Stathmin	3	50	5	29
				44	1	16
				43	2	10
	Q64332	Synapsin-2	2	36	2	2
				34	2	5
Rat	P41350	Caveolin-1	3	73	2	11
				64	2	11
	P45592	Cofilin-1	2	139	4	29
				84	2	20
	P07727	Glycine receptor alpha-1 chain precursor	1	34	1	1
	Q62968	Nav1.8	1	28	3	1

Accession numbers refers to the number used to archive the protein in the Swiss-Prot Database. The sequence coverage is expressed as the number of amino acids spanned by the assigned peptides divided by the sequence length. All animal experiments are carried out according to the local ethical committee at the Karolinska Institute (Application N282/06). Reproduced with permission from ref. 5

was retrieved in a significant manner in rat brain extracts. Furthermore, using SPR technology, we have shown a direct interaction between Nav1.8 (85–103) and Cav-1 (81–100). These findings provide evidence for the efficacy and the reliability of this protocol[5].

Cav-1 was also found in recovery experiments using Cav-1 (81–100) as an interacting partner in rat and mouse brain extracts. This was expected as Cav-1 is known to oligomerize[31]. Furthermore, an interaction between Cav-1 and stathmin was found with brain extracts from mouse. It is interesting to notice that Cav-1 and stathmin have been suggested earlier to interact in vascular smooth muscle cells[32].

The protocol presented here has been used for the successful identification of several partners to FKBP-12 (ref. 4) as well as a novel interaction between Nav1.8 and Cav-1 (ref. 5). These interactions should be confirmed with other methods such as SPR-based techniques and/or other biochemical approaches. However, this protocol is suitable for discovery-oriented screening for other protein–protein interactions.

Note: Supplementary information is available via the HTML version of this article.

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