

Binding & Kinetic Analysis of Protein Interactions Probed by Label-Free SpotMatrix SPR Technology in a Peptide Chip Format

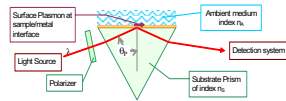
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Introduction

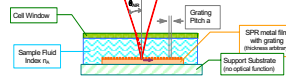
Protein-protein interactions play central roles in almost all cellular responses. Specific interaction between peptide motifs and signaling domains such as SH2 and WW domains is critical for a variety of signaling pathways. Many techniques have been developed to study protein binding to peptide motifs. These include ELISA, far western, immuno-precipitation or pull down assays. Applied Biosystems has developed a new platform that uses Grating-Coupled Surface Plasmon Resonance (GC-SPR) for kinetic measurement of molecular interactions between unlabeled analytes and biomolecules immobilized on an affinity chip. The optical design of this platform allows simultaneous affinity characterization of up to 400 targets spotted on a Gold Affinity Chip. We describe this SpotMatrix SPR technology and its application to protein-peptide interactions to illustrate how this technology is applied to the study of biomolecular interactions.

SpotMatrix SPR Technology

SPR Biosensor Optics



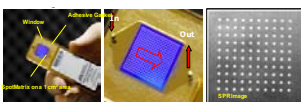
Kretschmann prism coupling. This configuration is commonly found in available SPR instrumentation.



Grating-coupled SPR. This configuration is used in the Applied Biosystems SpotMatrix SPR platform.

The **Kretschmann** configuration typically used in SPR instruments depends on a prism to measure SPR angles. In this configuration, incident light hits the gold layer on the opposite side of the immobilized biomolecules, posing strict requirements for the optical properties of the support substrate and the thickness of the gold layer. In the **grating-coupled** configuration, a fine grating on the chip surface provides optical coupling and allows imaging of the entire surface at once, enabling simultaneous real-time binding analysis at every spot on the surface. Here, incident light hits the gold layer from the top and through the biomolecular layer, avoiding the stringent need for an optical quality support substrate and specific gold layer thickness. The affinity chips are disposable.

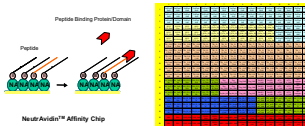
SPR SpotMatrix Platform



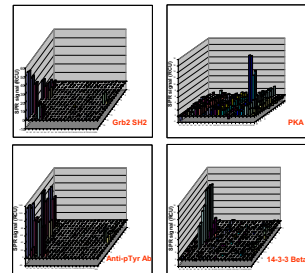
Working with a SpotMatrix SPR Platform. Left, Up to 400 targets are spotted onto a 1 cm² area of the affinity chip using standard floating-pin spotter. A 40 µl flow cell is then assembled over the SpotMatrix by attachment of a self-adhesive window containing a fluid inlet and outlet (center). The assembled affinity chip is then inserted into the instrument to measure the binding of an analyte to each of the immobilized targets using a CCD camera for imaging the entire surface in real-time (right).

Protein/Domain Binding to Peptide SpotMatrix

Experimental Design. Peptides were synthesized as 15- or 20-mers by Sigma-Genosys using their PEPscreenSM technology, a high-throughput peptide synthesis platform. Peptides contain an N-terminal biotin and a diethylene glycol linker between the biotin and first amino acid residues. The average peptide purity is 73% for 15mer and 61% for 20mer peptides. Biotinylated peptides were spotted onto a NeutrAvidin[™] Affinity Chip using a Cartesian spotter. Analytes, including full length proteins or domains, were flowed over the peptide SpotMatrix. Analyte binding to the peptides can be represented as end-point equilibrium binding (i.e. average of the last minute of binding signal before dissociation begins) as well as the affinity trace. Affinity constants, such as K_{on} (K_{on}), K_{off} (K_{off}) and K_D (K_D), were calculated using the Applied Biosystems 8500 Affinity Chip Analyzer data analysis software.



Peptide SpotMatrix. Left, schematic diagram of the assay. Right, layout of peptides spotted on the affinity chip. Over 50 different peptides representing a wide range of protein binding motifs were spotted on the affinity chip. Peptides represent binding motifs for SH2 domains, 14-3-3 proteins, Actin, WW domains and cAMP-dependent kinase. Three concentrations (2µM, 10µM, 50µM) for each peptide were spotted in duplicate.

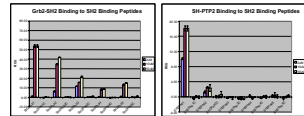


Binding Specificity of Analyte to Peptide SpotMatrix. Upper Left, the SH2 domain of Grb2 protein binds specifically to peptides containing phosphotyrosine. Upper right, protein kinase A catalytic subunit binds specifically to a peptide derived from protein kinase A inhibitor (PKI). Lower left, anti-phosphotyrosine antibody binds specifically to all the peptides containing phosphotyrosine but not nonphosphorylated peptides or phosphoserine or phosphothreonine peptides on the affinity chip. Lower right, protein 14-3-3 Beta binds specifically to peptide containing phosphothreonine and phosphoserine peptide with the cognate flanking sequence.

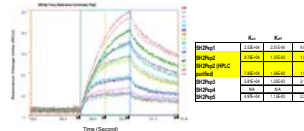
SH2 Domain-Peptide Interaction

Peptide	Sequence	Full protein
SH2Pep1	[Bio]q[DEG]GSGSGRPP[Py]r[YNV]EF	SH2 (Y177)
SH2Pep2	[Bio]q[DEG]GSSGSSGPP[Py]r[YNV]Q	SH2 (Y37)
SH2Pep3	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	SH2 (Y1068)
SH2Pep4	[Bio]q[DEG]GSGVGNP[Py]r[YNV]LTVQ	SH2 (Y1139)
SH2Pep5	[Bio]q[DEG]DFTLPP[Py]r[YNV]G	SH2 (Y1068)

Phosphotyrosine containing peptides in the SpotMatrix



Specificity of SH2 Domain Binding. Left, 100nM of SH2 domain from Grb2 was flowed over the peptide SpotMatrix. Right, 100nM of GST fusion of SH2-PTP2 protein was flowed over the peptide SpotMatrix. The maximum binding was observed with peptide spotted at 50 µM. The optimal binding motif for Grb2 SH2 domain is pYXX, which is present in all 5 SH2 peptides containing phosphotyrosine. The optimal binding motif for SH2 domain of SH-PTP2 is pY(V/I/V/I) is present in SH2Pep1 and SH2Pep2. Data above agree with this reported specificity. Peptides named SH2Pep1C, SH2Pep2C, SH2Pep3C, SH2Pep4C and SH2Pep5C are control peptides that have the same sequence as SH2Pep1, SH2Pep2, SH2Pep3, SH2Pep4 and SH2Pep5, respectively, but without a phosphate group on the Tyrosine residue.

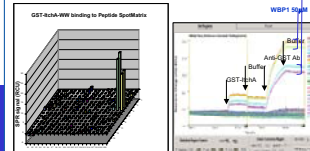


Kinetics Analysis of SH2 Binding. SH2-binding peptides were spotted on an affinity chip at concentrations ranging from 0.78µM up to 100µM. Left, affinity trace of SH2 domain of Grb2 binding to SH2Pep2 peptide is shown. Right, global fit of the affinity traces for Grb2-SH2 is shown. The kinetic data from the PEPscreenSM-synthesized SH2Pep2 peptide and a control (HPLC-purified) SH2Pep2 peptide is very similar.

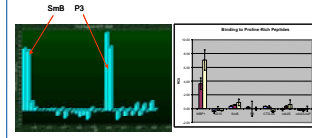
Proline-rich Peptide Interaction with WW and SH3 Domains

WW domain	WW domain binding peptide	Ref. source	Comments
WW1	[Bio]q[DEG]GSGSGRPP[Py]r[YNV]EF	1	SH2
WW2	[Bio]q[DEG]GSSGSSGPP[Py]r[YNV]Q	2	SH2
WW3	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	3	SH2
WW4	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	4	SH2
WW5	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	5	SH2
WW6	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	6	SH2
WW7	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	7	SH2
WW8	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	8	SH2
WW9	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	9	SH2
WW10	[Bio]q[DEG]GSSGPP[Py]r[YNV]G	10	SH2

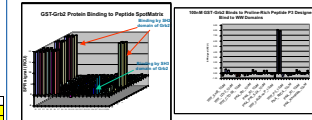
Proline-rich Peptides on the Affinity Chip. Proline-rich peptide motifs are known to bind to a number of protein domains, such as SH3 and WW domains. WW domains can be further subdivided into four groups based on their particular binding preferences. Above, peptides representing all four binding motifs, along with control peptide, were spotted onto the affinity chip.



WW Domain of ItchA Binds Specifically to WBP1 Peptide. 200nM of GST-ItchA WW domain was flowed over the peptide SpotMatrix. After washing with buffer, anti-GST antibody was used to "supershift" enhance the binding signal. Left, end-point signal of peptide SpotMatrix binding to ItchA is shown. Of over 50 different peptides, including some proline-rich peptides, only WBP1 peptide shows significant binding. Right, affinity trace of the binding to all proline-rich peptides is shown with the supershift assay.



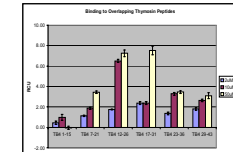
WW Domain Binding Specificity. Left, 5 µM WW domain of FBP21 Protein was flowed over the peptide SpotMatrix. Only Smb and P3 peptides give a binding signal with the supershift assay. Both peptides belong to the same subclass (Class III) of WW domain binding motifs. Right, 300nM of GST-Nedd4 WW domain was flowed over the peptide SpotMatrix. Only WBP1 peptide binds to the WW domain in a dose dependent manner with the supershift assay. The binding specificity of both FBP21 and Nedd4 agrees with literature reports (ref. MCB, 2001; 21(22):7617-7628).



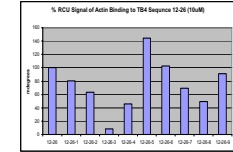
Cross-talk Between SH3 and WW Domains. 100nM of GST-Grb2 full length protein was flowed over the peptide SpotMatrix. Grb2 protein contains two SH3 domains separated by one SH2 domain. Left, GST-Grb2 protein not only binds to phosphotyrosine containing peptides, but also to the P3 proline-rich peptide, presumably through its SH3 domain. Right, a detailed data analysis from the same experiment shown at left. Among all proline-rich peptides spotted at 10µM, only P3 shows specific binding. Smb peptide, which is very homologous to P3 in sequence, shows no binding. The SH3 binding motif, [R/K]XXPPXp or PXXPP[R/K], is very homologous to P3 sequence. Binding of the Grb2 SH3 domain to Sam68, from which P3 peptide is derived, is supported by reports in the literature (J Cell Biochem. 2002; 86(1):99-106).

Mapping the Actin-Thymosin β4 Interaction

Thymosin β4 is a ubiquitous 43 amino acid polypeptide that is an important mediator of cell proliferation, migration, and differentiation. It is believed to be one the main G-actin sequestering peptides. It is also an anti-tumor target due to its important role in angiogenesis.



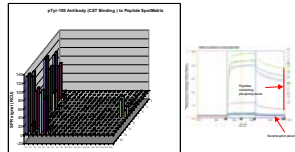
Mapping of the Actin-binding Site on Thymosin β4. Peptide positions within thymosin protein are indicated by their name. 8.7µM of monomeric G-actin was flowed over the peptide SpotMatrix. End-point binding of each individual overlapping peptide comprising thymosin is shown. Peptides 12-26 and 17-31 were shown to have the highest binding activity to actin.



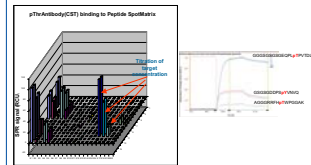
Determine Essential Residue in Thymosin for Actin Binding. A panel of sequential alanine substitution mutants of the peptide 12-26 were used to determine the essential residues for actin binding. All peptides were spotted at 10µM concentration. The bar graph above depicting the average end-point binding at equilibrium shows that the Lysine at position 18 (substituted with alanine in 12-26-3 mutant) of thymosin is essential for actin binding.

Modification-Specific Antibody Binding

Modification-specific antibodies are widely used by researchers studying signal transduction pathways. However, their affinity and specificity should be carefully evaluated. The SpotMatrix SPR platform is well-suited for this application.



Assessing Specificity and Affinity of a Phosphotyrosine Specific Antibody. 40nM of a phosphotyrosine specific antibody was flowed over the affinity chip. Left, end-point binding of phosphotyrosine specific antibody to the peptide SpotMatrix. Only those peptides containing phosphotyrosine show binding in the SPR assay. Right, representative affinity trace of the same experiment shown at left.



Assessing Specificity and Affinity of a Phosphothreonine Specific Antibody. 10nM of a phosphothreonine specific antibody was flowed over the affinity chip. Left, end-point binding of the antibody to the peptide SpotMatrix. Nonphosphorylated peptides and peptides containing phosphoserine show no binding. However, peptides containing either phosphothreonine or phosphotyrosine show significant binding. Right, representative affinity traces of the same experiment are shown.

Conclusions

- We describe the application of SpotMatrix SPR technology for the characterization of either full-length proteins or signal transduction domains, such as SH2, 14-3-3, WW and SH3, binding to over 50 different biotinylated peptides immobilized on a NeutrAvidin[™] Affinity Chip.
- Signaling domains show a high degree of specificity when binding to this peptide SpotMatrix.
- In a separate analysis, we used SpotMatrix SPR to interrogate the binding of Thymosin β4 to G-actin. A scan of overlapping peptides representing the entire Tβ4 protein was used to identify the actin-binding site, whereas an alanine scan was used to identify key residues for the interaction.
- We also show the application of this technology to assess specificity as well as affinity for capture agents such as modification-specific antibodies.
- By combining parallel screening with binding kinetics, this system improves the workflow and productivity for performing affinity screening and high throughput binding characterization studies.

Acknowledgments

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