Mass Spectrometry: Reverse Process for Synbody Discovery

&

Validation of Peptide Microarray Data: A Story of Landing Lights

by

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ABSTRACT

A synbody is a newly developed protein binding peptide which can be rapidly produced by chemical methods. The advantages of the synbody producing process make it a potential human proteome binding reagent. Most of the synbodies are designed to bind to specific proteins. The peptides incorporated in a synbody are discovered with peptide microarray technology. Nevertheless, the targets for unknown synbodies can also be discovered by searching through a protein mixture. The first part of this thesis mainly focuses on the process of target searching, which was performed with immunoprecipitation assays and mass spectrometry analysis. Proteins are pulled down from the cell lysate by certain synbodies, and then these proteins are identified using mass spectrometry. After excluding non-specific bindings, the interaction between a synbody and its real target(s) can be verified with affinity measurements. As a specific example, the binding between 1-4-KCap synbody and actin was discovered. This result proved the feasibility of the mass spectrometry based method and also suggested that a high throughput synbody discovery platform for the human proteome could be developed.

Besides the application of synbody development, the peptide microarray technology can also be used for immunosignatures. The composition of all types of antibodies existing in one's blood is related to an individual's health condition. A method, called immunosignaturing, has been developed for early disease diagnosis based on this principle. CIM10K microarray slides work as a platform for blood antibody detection in immunosignaturing. During the analysis of an immunosignature,

the data from these slides needs to be validated by using landing light peptides. The second part of this thesis focuses on the validation of the data. A biotinylated peptide was used as a landing light on the new CIM10K slides. The data was collected in several rounds of tests and indicated that the variation among landing lights was significantly reduced by using the newly prepared biotinylated peptide compared with old peptide mixture. Several suggestions for further landing light improvement are proposed based on the results.

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ABBREVIATIONS

AMI	Applied Microarrays, Inc
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CIM	Center for Innovations in Medicine
CV	Coefficient of Variation
DMF	Dimethylformamide
HPLC	High Pressure Liquid Chromotography
IP	Immunoprecipitaion
MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
PBS	Phosphate-Buffered Saline
PBST	Phosphate-Buffered Saline with Tween-20
PMT	Photomultiplier Tube
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween-20
TFA	Trifluoroacetic Acid

TNF-α	Tumor Necrosis Factor-α

CHAPTER 1

MASS SPECTROMETRY: REVERSE PROCESS FOR SYNBODY DISCOVERY

1.1 Introduction

1.1.1 Synbody

Recently, the concept of '-omics' has been emphasized in the biological sciences and many protein binding reagents will be needed to develop these '-omics' fields. Antibodies are widely used for detecting and identifying specific intracellular or extracellular proteins in biological assays. The properties of antibodies also allow them to be useful for diagnosis and clinical treatments. However, the procedures of antibody production are time-consuming and expensive. The most widely used method for producing antibodies involves injecting specific antigens into mammals (mouse, rabbit, goat, etc.) and then isolating antibody containing serum from the animals. Monoclonal antibodies are even more difficult to produce since the hybridoma technology is required. Developing a method to develop protein binding reagents quickly is necessary and urgent[1-4]. Strategies for *in vitro* protein binding peptide selection, such as mRNA and phage display, have been reported[5-7].

Small peptides, such as 20mers consisting of a random sequence, are good candidates for protein binding reagents. They can be synthesized in large scale by chemical methods, which are much cheaper than *in vivo* antibody production approaches. Also, with the increasingly mature peptide microarray technology, the selection of potential protein binders by high throughput array screening is relatively easy now[8-10]. Considering the size and flexibility of small peptides, we don't expect a high binding affinity between a 20mer and its target protein. However, the affinity issue can be solved theoretically by the strategy of linking two or more 20mer peptides together. This new type of protein binding reagent has been given the title of "synbody". If the binding affinity of two peptides selected for the same protein target is Kd_1 and Kd_2, respectively, then the affinity of the synbody which is made of the two peptides combined can be calculated by the following equation:

$$Kd = Kd_1 \times Kd_2.$$

There are two strategies for synbody discovery: a forward strategy which involves designing synbodies for specific protein targets[2, 11]; a backward strategy which involves searching binding targets for existing synbodies[12]. The forward strategy is mainly based on the peptide microarray technology. For example the protein

tumor necrosis factor-α (TNF-α) can be screened on a 10K random peptide microarray in order to select good protein surface binders. Each of the selected peptide is unique in sequence and should bind to different areas of the target. Synbodies are made by linking these peptides in random combinations. Subsequent measurements can further select synbodies with high binding affinity. Although both peptide arms are good surface binders, most of the synbodies lose the affinity for the original target after the two peptides are connected. However, the proteins which the synbody has affinity for can be discovered using the backward strategy. The synbodies are applied to protein arrays which contain a large known human protein library. Potential targets can be revealed by array data analysis[13]. Additionally, a synbody can capture its target by an immunoprecipitation assay followed by mass spectrometry detection to identify the specific protein[12, 14]. Most of my work has focused on the immunoprecipitation aspect.

1.1.2 Immunoprecipitation and Mass Spectrometry

The synbodies are biotinylated so that they can be captured by the streptavidin linked to the surface of the magnetic beads in the immunosignature assay. During the incubation with human cell lysate, synbodies may capture their potential target. After the synbody-protein complexes are separated, the samples are run in an SDS-PAGE gel and

interesting bands are cut out of the gel and used for mass spectrometry. The complexes can also be digested directly on the beads (Figure 1.1). The target proteins are digested by trypsin and the peptides mixture can be identified using mass spectrometry. By searching the peptide information in certain databases, the protein which contains the peptides resulting from digestion can be determined (Figure 1.2)[15-17].

Figure 1.1
Reverse process for synbody discovery.

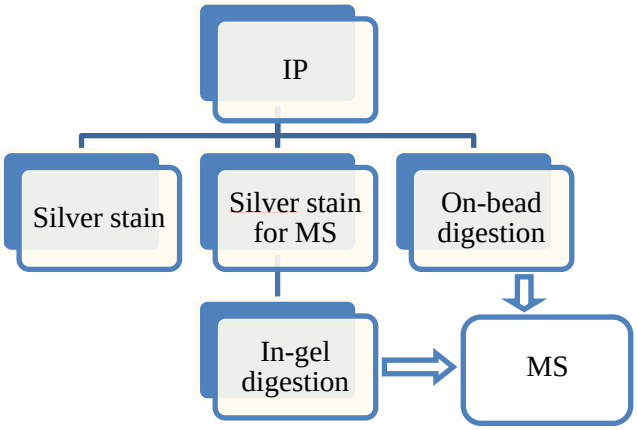
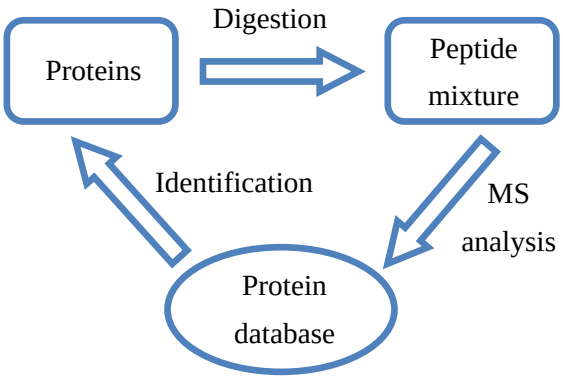


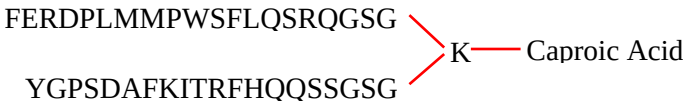
Figure 1.2
Protein identification by mass spectrometry.



1.1.3 1-4-KCap, Actin, and Related Proteins

The synbody 1-4-KCap mainly consists of two peptides, TNF-α-1 and TNF-α-4. Both of the peptides are 20mers and their sequences are FERDPLMMPWSFLQSRQGSG (1) and YGPSDAFKITRFHQQSSGSG (4). These two peptides are connected to the two amino groups on the same lysine. A caproic acid is linked to the carboxyl group of the lysine. The structure of 1-4-KCap synbody is shown in Figure 1.3.

Figure 1.3
Abbreviated structure of 1-4-KCap synbody.



Actins are proteins with a molecular weight around 42kDa which are highly conserved across all species. The microfilament (F-actin) has a two-stranded helix structure which is formed by polymerization of global actin (G-actin). Since the microfilament is one of the major components of the cytoskeleton, actin plays essential roles in cellular processes such as cell motility and division[18]. Another important part of the cytoskeleton are microtubules which are assembled by α-tubulin and β-tubulin. Both of the two subtypes of tubulin have a molecular weight around 55kDa[19]. Study of the cytoskeletal proteins has revealed the interaction between actin and tubulin[20]. Myosin is a family of motor proteins which is only found in eukaryotic cells. These

motor proteins are actin binding proteins and have a function of motility based on microfilaments[21]. Glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and lactate dehydrogenase are glycolytic enzymes which are involved in the process of glycolysis. The glycolytic enzymes can form a protein complex and interact with both microfilaments and microtubules[22-24].

1.2 Materials and Methods

1.2.1 Biotinylation

Reagents and Materials

EZ-link NHS-PEG_4-Biotin (Thermo #21329); EZ-link Amine-PEG_2-Biotin (Thermo #21346); EZ-link Maleimide-PEG_2-Biotin (Thermo #21902); MICROCON® Centrifugal Filter Devices Ultracel YM-3 (Millipore #42404).

Equipment

Autoflex/Microflex MALDI TOF mass spectrometer (Bruker).

Solution Preparation

*TCEP:*100mM (28.5mg/ml) TCEP (tris(2-carboxyethyl)phosphine) in 1X TRIS (tris(hydroxymethyl)aminomethane) buffer (pH 8.5); *Biotin:*20mM biotin in 1XPBS (for carboxyl group (-COOH), add 267µl of PBS to 2mg of Amine-PEG_2-Biotin; for amino

group (-NH_2) reaction, add 170µl of PBS to 2mg of NHS-PEG_4-Biotin; for sulfhydryl group (-SH) reaction, add 190µl of PBS to 2mg of Maleimide-PEG_2-Biotin); *MALDI matrix:*Spatula tip α-cyano, 166µl 100% acteonitrile, 332µl water, and 40µl 10% TFA.

Procedures

A. TCEP reduction to remove protecting group StBu

Add the proper amount of prepared *TCEP* into synbody to make a 20mM solution of TCEP. React the mix at 50°C for 30 minutes or at room temperature overnight. Check the molecular weight by MALDI (removal of StBu will cause a decrease in mass of 89Da).

B. Biotinylation

Adjust the pH of the solution containing synbody to 6.5-7.5. Add proper amount of *Biotin* to the synbody solution to make a 20:1 mole ratio of biotin to synbody. React at room temperature overnight. Check the molecular weight by MALDI (addition of biotin will cause an increase in mass of 372.50Da for Amine-PEG_2-Biotin, 473.22Da for NHS-PEG_4-Biotin, and 525.62Da for Maleimide-PEG_2-Biotin). If the synbody is not completely biotinylated, then add another 20 mole equivalents of biotin to synbody for 2 hours. Dilute synbody to 500µl with 18.2MΩ water. Spin at 13.4kRPM (12,100×g) on a

3kDa spin filter at room temperature for 30 minutes. Flip the filter into a new tube and spin at 5kRPM (1,680×g) for 5 minutes to collect the synbody. Check the purity of the biotinylated synbody by MADLI again. Measure the concentration of biotinylated synbody by NanoDrop (Protein A280).

1.2.2 Cell Lysis

Reagents and Materials

A549, MCF7, and MCF10A cell lines from ATCC®; Protease Inhibitor Cocktail (100X) (Thermo #78410); Halt Phosphatase Inhibitor Cocktail (100X) (Thermo #78420); 20% Triton X-100.

Solution Preparation

*Cell Lysis Solution:*10µl of 100X Protease Inhibitor, 10µl of 100X Phosphatase Inhibitor, 50µl of 20% Triton X-100, and 930µl of 1XPBS for 1ml cell lysis buffer.

Procedures

Prepare the *Cell Lysis Solution* and keep it on ice for 10 minutes before use. Add the *Cell Lysis Solution* to human cells and shake at 4°C for 30 minutes. Transfer the solution to microfuge tubes and centrifuge at 13.4kRPM (12,100×g) for 10 minutes. Take out the supernatant for use.

1.2.3 BCA

Reagents and Materials

Pierce® BCA Protein Assay Kit (Thermo# 23225); BCA Reagent A; BCA Reagent B; Albumin Standard Ampules, 2mg/ml.

Equipment

SpectraMax 190 Absorbance Microplate Reader (Molecular Devices).

Solution Preparation

*BSA Standards:*Dilute 2.0mg/ml Albumin Standard to 1.5mg/ml, 1.0mg/ml, 0.75mg/ml, 0.5mg/ml, 0.25mg/ml, and 0.125mg/ml. *Working Reagent:*Mix 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B).

Procedures

A. Microplate Procedure

Loading sample (in triplicate);

Blank: add 10µl PBS into each well;

Standard: add 10µl *BSA Standards* (from 2mg/ml to 0.125mg/ml) into each well.

Protein with unknown concentration: add 10µl unmeasured sample into each well (1µl sample in 9µl PBS, i.e. 1:10 dilution of the sample).

Add 200µl *Working Reagent* into each well. Incubate at 37°C for 30 minutes. Measure absorbance at 562nm by SpectraMax 190 Absorbance Microplate Reader.

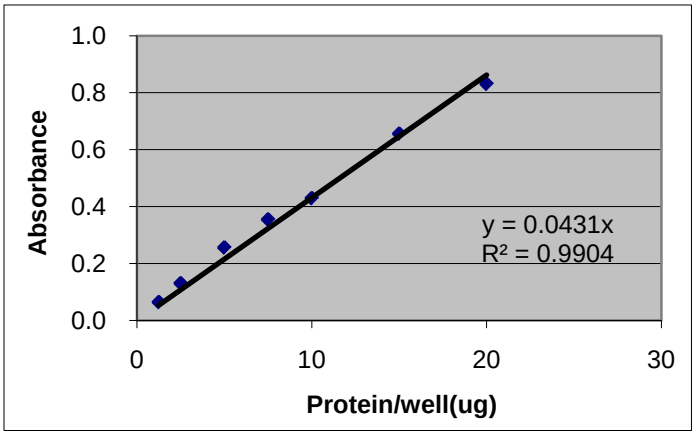
B. Concentration calculation (example)

Apply the data from SpectraMax to Table 1.1. Make a scatter chart with the data in grey cells and generate the equation by linear fitting (Figure 1.4). Therefore, the concentration of the sample can be calculated by the absorbance.

Table 1.1
Concentration calculation table for BCA reaction.

	Blank	A	B	C	D	E	F	G	Sample
1	0.0979	0.9532	0.7121	0.4950	0.4350	0.3319	0.2186	0.1431	0.2764
2	0.0927	0.9331	0.7954	0.5421	0.4782	0.3563	0.2240	0.1601	0.2643
3	0.0957	0.8921	0.7466	0.5380	0.4379	0.3672	0.2367	0.1776	0.2653
average	0.0954	0.9261	0.7514	0.5250	0.4504	0.3518	0.2264	0.1603	0.2687
average - blank	0.0000	0.8307	0.6559	0.4296	0.3549	0.2564	0.1310	0.0648	0.1732
prot/well(µg)	0	20	15	10	7.5	5	2.5	1.25	4.0193

Figure 1.4
Linear fitting for concentration calculation.



1.2.4 Immunoprecipitation

Reagents and Materials

Dynabeads® M-280 Streptavidin (Invitrogen™ #112-05D/112-06D); MagnaBind Magnet for 96-Well Plate Separator (Thermo #21358); Actin from bovine muscle (Sigma #A3653); Tubulin, porcine (Sigma #T6954); Myosin from rabbit muscle (MP #02153887).

Solution Preparation

PBST (0.05%): 1XPBS with 0.05% tween-20. *PBST (0.1%):* 1XPBS with 0.1% tween-20.

Procedures

A. Prewash of the Dynabeads

Mix the beads and add 100µl of the beads into each microfuge tube. Use the dynal stand to remove liquid. Wash the beads with 1ml *PBST (0.05%)* for three times.

B. Synbody binding to beads

Remove the liquid and add 1µM synbody in 100µl *PBST (0.05%)* to the beads. Mix the solution and shake at room temperature for 2 hours. Use the dynal stand to remove liquid. Wash the beads with 1ml *PBST (0.05%)* for three times.

C. Protein pull-down

Remove the liquid and add desired amount of protein in 100µl *PBST (0.05%)* to the beads. Incubate at 4°C overnight with gentle shaking. Wash the beads with 1ml *PBST (0.1%)* for five times (between each wash, mix the beads and wait for 10 minutes). Wash the beads with 1ml 18.2MΩ water.

1.2.5 SDS-PAGE

Reagents and Materials

NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm, 10 well (Invitrogen™ # NP0321BOX); NuPAGE® Sample Reducing Agent (10X) (Invitrogen™ #NP0004); NuPAGE® LDS Sample Buffer (4X) (Invitrogen™ #NP0007).

Solution Preparation

*1X sample buffer (1ml):*100µl 10X reducing buffer, 250µl 4X NuPAGE LDS sample buffer, and 650µl 1X PBS.

Procedures

Remove the liquid from the previous step and add 20µl 1X sample buffer to the beads. Incubate at 70°C for 10 minutes. Cool the samples and centrifuge at 13.4kRPM

(12,100×g) for 2 minutes. Use the dynal stand to load the sample on a protein gel. Run the gel in 1X MOPS buffer with 200V for 1 hour. Process gel to stain or digest.

1.2.6 Silver Stain

Reagents and Materials

Pierce® Silver Stain Kit (Thermo #24612); Pierce® Silver Stain for Mass Spectrometry (Thermo #24600).

Equipment

ChemiDoc™ XRS+ System.

Solution Preparation

Fixing Solution: 30% ethanol : 10% acetic acid in ultrapure water; *Sensitizer Working Solution:* 40µl Sensitizer with 20ml ultrapure water; *Stain Working Solution:* 400µl Enhancer with 20ml Stain; *Developer Working Solution:* 400µl Enhancer with 20ml Developer.

Procedures

Wash gel with ultrapure water, twice. Fix the gel with *Fixing Solution* for 15 minutes, twice. Wash the gel with 10% ethanol for 5 minutes, twice. Wash the gel with ultrapure water for 5 minutes, twice. Sensitize the gel with *Sensitizer Working Solution*

for 1 minute. Wash the gel with ultrapure water for 1 minute, twice. Stain the gel with *Stain Working Solution* for 30 minutes. Wash the gel with ultrapure water for 20 seconds, twice. Develop the gel with *Developer Working Solution* for 2~3 minutes until bands appear. Stop with 5% acetic acid for 10 minutes. Take pictures by ChemiDoc™ XRS+ System.

1.2.7 In-Gel Digestion

Reagents and Materials

SilverSNAP® Stain for Mass Spectrometry (Thermo #24600) (Silver Destain Reagent A; Silver Destain Reagent B); In-Gel Tryptic Digestion Kit (Thermo #89871) (Trypsin, Modified; Trypsin Storage Solution; Acetonitrile; Ammonium Bicarbonate; Tris(2-carboxyethyl)phosphine (TCEP); Iodoacetamide (IAA)); Trifluoroacetic Acid or Formic Acid.

Solution Preparation

Destain Solution: 74µl of Destain Reagent A, 245µl of Destain Reagent B and 4ml of ultrapure water (use the solution within the same day); *Wash Solution:* 25mM ammonium bicarbonate, 50% acetonitrile (store at 4°C); *Digestion Buffer:* 25mM ammonium bicarbonate (store at 4°C); *Reducing Buffer:* 3.3µl of TCEP with 30µl of

Digestion Buffer for each gel piece (prepare just before use); *Alkylation Buffer:* 100mM IAA in Digestion Buffer (prepare just before use in foil-wrapped tubes); *Trypsin Stock:* 20µg modified trypsin in 20µl of Trypsin Storage Solution (store at -20°C, ~5µl for each aliquot); *Trypsin Working Solution:* dilute *Trypsin Stock* 10-fold by adding 45µl of ultrapure water (store at -20°C); *Activated Trypsin:* dilute 1µl of *Trypsin Working Solution* with 9µl of Digestion Buffer for each sample being processed (prepare just before use).

Procedures

A. Excising and Destaining Gel Pieces

Wash the stained gel with ultrapure water for 10 minutes, twice. Cover the light box with cling wrap and excise protein band with a clean scalpel on the box. From a blank region of the gel, excise another gel piece of the same size to use as a control sample. Place gel pieces in clean 0.5ml microcentrifuge tubes. Add 0.2ml of the *Destain Solution* to the gel pieces, mix gently and incubate at RT for 15 minutes, twice. Remove the *Destain Solution* and wash gel pieces with 0.2ml of Wash Solution for 10 minutes, three times. Store the gel pieces overnight at -20°C, or proceed with the in-gel tryptic digestion steps.

B. Reduction and Alkylation

Remove Wash Solution, add 30µl of *Reducing Buffer* to the tube and incubate at 60°C for 10 minutes. Allow samples to cool; then remove *Reducing Buffer*. Add 30µl of *Alkylation Buffer* to the tube and incubate samples in the dark at RT for 1hour. Remove *Alkylation Buffer*. Wash the sample with 200µl *Wash Solution* at 37°C for 15 minutes with shaking, twice.

C. Digestion

Remove *Wash Solution*. Shrink each gel piece with 50µl of acetonitrile for at RT 15 minutes. Carefully remove acetonitrile and allow gel pieces to air-dry for 5-10 minutes. Swell gel pieces with 10µl of *Activated Trypsin* solution at RT for 15 minutes. (If 10µl is insufficient to cover and fully swell gel pieces, increase the volume accordingly.) Add 25µl *Digestion Buffer* to the tube. Incubate samples at 37°C for 4 hours or at 30°C overnight with shaking. Remove digestion mixture and place in a clean tube. To further extract peptides, add 10µl 1% trifluoroacetic acid or 1% formic acid solution to gel pieces and incubate for 5 minutes. Remove extraction solution and add to digestion mixture from the previous step. (This step is optional. The final samples will be diluted.) Sample is now ready for liquid chromatographic separation and electrospray ionization mass

spectrometry (LC-ESI MS) or for additional processing/clean-up as required for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or nanospray ionization mass spectrometry.

1.2.8 On-Bead Digestion

Reagents and Materials

In-Solution Tryptic Digestion Kit and Guanidination Kit (Thermo #89895) (Trypsin, Modified; Trypsin Storage Solution; Ammonium Bicarbonate; No-Weigh™ DTT; Iodoacetamide (IAA)); Trifluoroacetic Acid (TFA).

Solution Preparation

Digestion Buffer: 50mM ammonium bicarbonate (store at 4°C); *Reducing Buffer:* 100mM DTT (resuspend the No-Weigh™ DTT with 500µl of ultrapure water); *Alkylation Buffer:* 100mM IAA in Digestion Buffer (prepare just before use in foil-wrapped tubes); *Trypsin Stock:* 20µg modified trypsin in 20µl of Trypsin Storage Solution (store at -20°C, ~5µl for each aliquot); *Activated Trypsin:* 100ng/µl Trypsin (dilute *Trypsin Stock* 10-fold by adding 45µl of ultrapure water).

Procedures

A. Reduction and Alkylation

Wash the beads from immunoprecipitation with ultrapure water once. Discard the water. Add 15µl of Digestion Buffer and 1.5µl of Reducing Buffer to each sample (beads). Add 10.5µl of ultrapure water to make a final volume of 27µl for each sample. After incubating samples at 95°C for 5 minutes, allow samples to cool. Add 3µl of Alkylation Buffer to each tube and incubate in the dark at RT for 20 minutes.

B. Digestion

Add 1µl of Activated Trypsin to the reaction tube and incubate at 37°C for 3 hours. Add an additional 1µl of Activated Trypsin to the reaction tube and incubate at 37°C for 2 hours. (Or incubate reaction overnight at 30°C.) Add 6µl of TFA to stop reaction, or proceed directly to sample preparation for further analysis.

1.2.9 Western Blot

Reagents and Materials

Nitrocellulose/Filter Paper Sandwiches (Bio-Rad #162-0233); NuPAGE® Transfer Buffer (20X) (Invitrogen™ #NP0006-1).

Equipment

Typhoon Trio+ Variable Mode Imager (GE Healthcare).

Procedures

Transfer proteins from the SDS-PAGE gel to the membrane in Transfer Buffer, 100V, 0.35A, for 1 hour. Wash the membrane with PBST for 5 minutes. Block the membrane in PBST with 1% BSA at room temperature for 1.5 hours. Incubate the membrane with the biotinylated synbody diluted in 1% BSA at 4°C overnight. Wash with PBST for 10 minutes, 4 times. Incubate with streptavidin conjugated Alexa Fluor 488 dye diluted in 1% BSA at room temperature for 1 hour. Wash with PBST for 10 minutes, 5 times. Dry the membrane with filter paper and scan by Typhoon with proper settings.

1.3 Results and Discussions

1.3.1 Biotinylation

Before applying the synbodies to immunoprecipitation, the linkers between two peptide arms were biotinylated (work done by Miti Shah from CIM). Therefore, the synbodies can be captured or detected easily by the strong binding between biotin and streptavidin[25, 26]. A successful biotinylation was detected by measuring the molecular weight through MALDI. A difference of molecular weight around 372Da was observed (data not shown).

1.3.2 Immunoprecipitation Procedure Optimization

In the immunoprecipitation assay, the biotinylated synbody was attached to the streptavidin magnetic beads and used for capturing potential targets from the cell lysate. We tried to optimize the procedure of the experiment in order to increase the amount of target protein pulled down and reduce non-specific binding. Part of the work (optimizing the amount of synbody and cell lysate used in the assay) was done by Miti Shah and Clarissa Anderfer-Lopez from CIM. Due to the sticky surface of the beads (the part of the surface which wasn't streptavidin covered), some non-specific binding proteins were also pulled down from the lysate. I tested the last wash after pull-down with several time and detergent concentrations (Figure 1.5). The acid wash was also tested and sent to the mass spectrometry instrument for protein composition analysis (Table 1.2A and Table 1.2B). There was no significant difference shown for the composition of proteins obtained by

Figure 1.5
Wash time and detergent concentration test. (100µl of beads, 250nM of synbody 26-23-KK-biotin, 100µg of human lysate A549, Silver Stain)

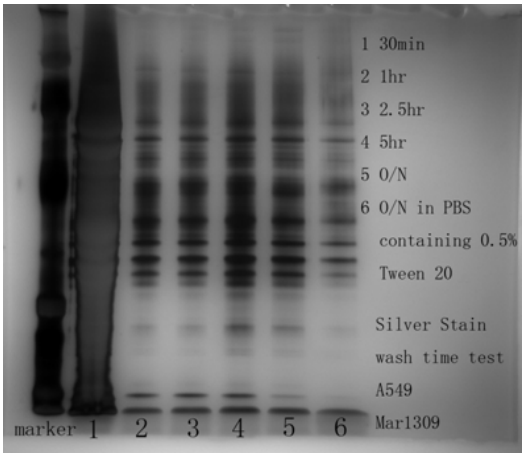


Table 1.2A

Human proteins identified by MS from on-bead digestion sample of 1-4-KCap pull-down with acid wash. (NC_B, negative control with amine-PEG_2-biotin blocking the surface of the beads and general wash; NC & PD, negative control and pull-down with general wash; NC_W1 & PD_W1, negative control and pull-down with pH=1.5 glycine wash; NC_W2 & PD_W2, negative control and pull-down with pH=2 glycine wash; NC_W3 & PD_W3, negative control and pull-down with pH=3 glycine wash.)

	NC_B	NC	PD	NC_W1	PD_W1	NC_W2	PD_W2	NC_W3	PD_W3	Database Accession#	Protein Name
# spectra	2	1	8	2	7	2	7	2	9	P63261	Actin, Cytoplasmic 2
mean intensity	9.17E+07	1.17E+08	4.11E+08	5.09E+07	1.48E+08	3.96E+07	2.40E+08	1.29E+08	2.37E+08		
# spectra	0	0	7	0	6	2	5	1	5	P35579	Myosin-9
mean intensity	0.00E+00	0.00E+00	1.59E+08	0.00E+00	8.27E+07	1.29E+07	9.10E+07	1.68E+07	1.15E+08		
# spectra	1	0	8	0	6	0	11	0	10	P07437	Tubulin beta chain
mean intensity	4.01E+07	0.00E+00	2.24E+08	0.00E+00	1.14E+08	0.00E+00	1.43E+08	0.00E+00	1.46E+08		
# spectra	1	3	5	4	8	4	6	3	4	P04406	Glyceraldehyde-3-phosphate dehydrogenase
mean intensity	7.24E+07	1.85E+07	3.20E+08	2.95E+07	9.97E+07	4.18E+07	2.21E+08	7.28E+07	2.13E+08		
# spectra	9	1	2	4	1	1	2	2	0	P04264	Keratin, type II cytoskeletal 1
mean intensity	1.10E+08	1.07E+08	1.05E+08	6.93E+07	1.71E+08	8.11E+07	1.46E+08	7.90E+07	0.00E+00		
# spectra	1	0	5	2	5	2	4	2	7	P68363	Tubulin alpha-1B chain
mean intensity	1.80E+07	0.00E+00	2.36E+08	2.39E+07	1.12E+08	2.94E+07	1.87E+08	5.78E+07	1.38E+08		
# spectra	0	0	2	0	1	0	4	0	4	P08238	Heat shock protein HSP 90-beta
mean intensity	0.00E+00	0.00E+00	1.44E+08	0.00E+00	3.94E+07	0.00E+00	1.24E+08	0.00E+00	1.40E+08		
# spectra	3	4	5	2	5	5	6	5	4	P02768	Serum albumin
mean intensity	2.23E+08	1.50E+08	4.16E+08	3.68E+08	3.08E+08	2.46E+08	2.55E+08	3.43E+08	2.87E+08		
# spectra	0	0	3	0	4	0	3	0	3	P14618	Pyruvate kinase isozymes M1/M2
mean intensity	0.00E+00	0.00E+00	1.61E+08	0.00E+00	4.29E+07	0.00E+00	1.14E+08	0.00E+00	1.20E+08		
# spectra	0	1	8	0	6	0	5	1	4	P00338	L-lactate dehydrogenase A chain
mean intensity	0.00E+00	5.58E+07	1.64E+08	0.00E+00	1.22E+08	0.00E+00	1.30E+08	1.44E+07	1.54E+08		

Table 1.2B
Human proteins identified by MS from on-bead digestion sample of 1-4-KCap pull-down with acid wash. (BK0~9, wash solution collected between each sample shown in Table 1.2A.)

BK0	BK1	BK2	BK3	BK4	BK5	BK6	BK7	BK8	BK9	Database Accession#	Protein Name
5 6.02E+06	1 4.57E+06	1 6.30E+06	5 2.53E+07	2 9.86E+06	3 1.28E+07	1 1.39E+07	3 9.71E+06	2 1.86E+07	4 1.28E+07	P63261	Actin, Cytoplasmic 2
0 0.00E+00	0 0.00E+00	0 0.00E+00	2 8.83E+06	0 0.00E+00	1 7.80E+06	0 0.00E+00	0 0.00E+00	1 6.02E+06	1 3.07E+06	P35579	Myosin-9
0 0.00E+00	0 0.00E+00	0 0.00E+00	1 3.43E+07	0 0.00E+00	4 9.29E+06	0 0.00E+00	3 1.31E+07	0 0.00E+00	4 1.45E+07	P07437	Tubulin beta chain
0 0.00E+00	0 0.00E+00	1 7.66E+06	4 3.25E+07	4 1.04E+07	3 1.51E+07	3 8.87E+06	5 1.76E+07	4 2.16E+07	3 2.44E+07	P04406	Glyceraldehyde-3-phosphate dehydrogenase
0 0.00E+00	2 4.20E+06	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	1 8.34E+06	0 0.00E+00	1 6.88E+06	P04264	Keratin, type II cytoskeletal 1
0 0.00E+00	0 0.00E+00	0 0.00E+00	3 2.63E+07	1 1.02E+07	2 1.16E+07	2 7.07E+06	3 1.64E+07	1 1.76E+07	4 1.19E+07	P68363	Tubulin alpha-1B chain
0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	0 0.00E+00	P08238	Heat shock protein HSP 90-beta
0 0.00E+00	2 1.33E+07	3 1.15E+07	1 1.12E+07	0 0.00E+00	0 0.00E+00	2 4.86E+06	0 0.00E+00	1 8.14E+06	0 0.00E+00	P02768	Serum albumin
1 8.85E+06	0 0.00E+00	0 0.00E+00	2 2.02E+07	0 0.00E+00	1 1.21E+07	0 0.00E+00	1 1.09E+07	0 0.00E+00	0 0.00E+00	P14618	Pyruvate kinase isozymes M1/M2
0 0.00E+00	0 0.00E+00	0 0.00E+00	2 6.20E+06	0 0.00E+00	1 2.41E+06	0 0.00E+00	1 6.62E+06	2 4.59E+06	1 6.39E+06	P00338	L-lactate dehydrogenase A chain

pull-down assay either in the SDS-PAGE gel or the mass spectrometry data.

For the purpose of adapting immunoprecipitation to high throughput synbody discovery, I also tested low amounts of beads and wash buffer with a 96-well plate as a platform for pull-down. The results showed that, by using 25μl of beads, a similar pattern of protein bands could be generated in the gel compared with using 100μl (data not shown). Moreover, the 96-well plate and magnetic separator worked as well as microfuge tubes and the dynal stand. This result demonstrated the feasibility of an automatic high throughput immunoprecipitation assay for synbody discovery.

1.3.3 1-4-KCap Synbody, Actin and Related Proteins

Two peptides, named as TNF-α-1 and TNF-α-4, were selected by peptide microarray screening. These peptides were good surface binders for TNF-α. Several synbodies were produced by combining these two peptides with different linkers. Figure 1.6 shows the pull-down result of 5 TNF-α-1, 4-peptide based synbodies. A similar pattern of protein bands was generated by three synbodies, while few proteins were pulled down by the other two. From the three synbodies which exhibited positive results during the pull-down assay, we chose 1-4-KCap which contains a relatively short linker

(lysine - caproic acid) for further study. The 1-4-KCap pull-down assay was repeated with a negative control (without synbody) (Figure 1.7).

Figure 1.6
IP for synbodies with different linkers. (100µl of beads, 250nM of synbody, 100µg of human lysate A549, Silver Stain; red arrow – potential actin band.)

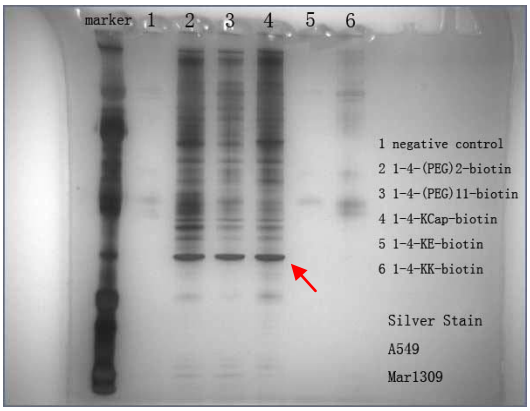
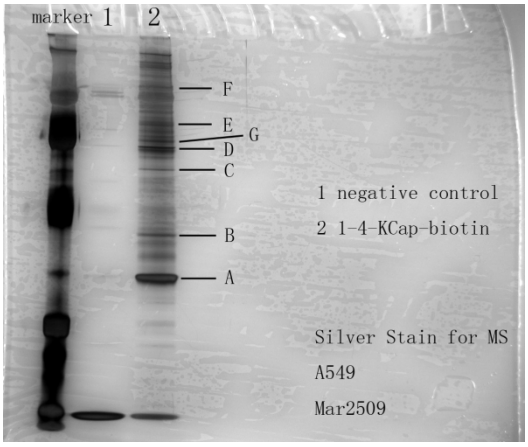


Figure 1.7
IP for 1-4-KCap-Biotin against human lysate A549. Labeled bands were identified by the mass spectrometry. (100µl of beads, 250nM of synbody, 100µg of human lysate A549, Silver Stain for MS)



Seven bands in lane 2 were chosen for an in-gel digestion test. These bands were either significantly darker than the corresponding bands in lane 1 or not have any corresponding

bands in this negative control lane. The bands were cut from the gel and processed for mass spectrometry analysis. Through protein database searching, the major proteins which existed in each gel piece were identified. The protein information is summarized in Table 1.3.

Table 1.3
The summary of human proteins identified by MS from in-gel digestion sample of 1-4-KCap pull-down (see Figure 1.6). According to UniProtKB database, most of the proteins are associated with Actin, which is contained in band A.

Band	Protein	Type
A	Actin, cytoplasmic 2	subunit
B	Vimentin	Actin-binding
C	Gelsolin	Actin-binding
D	Alpha-actinin-4	Actin-binding
E	Keratin, type II cytoskeletal 1	Actin-binding
F	Spectrin beta chain, brain 1	Actin-binding
G	Spectrin alpha chain, brain 1	Actin-binding

The result shown in Table 1.3 indicates that Actin might be a potential target for 1-4-KCap synbody. However, more evidence was still needed to prove the reproducibility of our result. Therefore, we repeated the 1-4-KCap pull-down assay and directly digested the protein complex on-bead. The mass spectrometry result after searching against a human protein database is shown in Table 1.4. Summary of the protein composition is also listed in Table 1.5. By checking the relationship among all the proteins listed in Table 1.5, we may further confirm the binding between our synbody and

Table 1.4
Human proteins identified by MS from on-bead digestion sample of 1-4-KCap pull-down. (NC, negative control; PD, pull-down; BK0~2, wash solution collected between each sample shown.)

	NC	PD	BK0	BK1	BK2	Database Accession#	Protein Name
# spectra	1	6	1	1	4	P63261	Actin, Cytoplasmic 2
mean intensity	4.67E+07	2.85E+08	1.85E+07	1.74E+07	1.67E+07		
# spectra	0	6	0	0	1	P14618	Pyruvate kinase isozymes M1/M2
mean intensity	0.00E+00	1.78E+08	0.00E+00	0.00E+00	8.75E+06		
# spectra	0	4	3	1	5	P04406	Glyceraldehyde-3-phosphate dehydrogenase
mean intensity	0.00E+00	2.28E+08	1.20E+07	1.12E+07	1.81E+07		
# spectra	0	4	3	1	3	P68363	Tubulin alpha-1B chain
mean intensity	0.00E+00	1.93E+08	7.32E+06	8.05E+06	1.18E+07		
# spectra	0	5	1	0	0	P07437	Tubulin beta chain
mean intensity	0.00E+00	1.44E+08	7.15E+06	0.00E+00	0.00E+00		
# spectra	4	5	1	3	1	P02768	Serum albumin
mean intensity	7.49E+08	5.13E+08	1.44E+07	1.14E+07	1.67E+07		
# spectra	0	2	0	0	0	P35579	Myosin-9
mean intensity	0.00E+00	1.35E+08	0.00E+00	0.00E+00	0.00E+00		
# spectra	0	0	1	1	1	P04264	Keratin, type II cytoskeletal 1
mean intensity	0.00E+00	0.00E+00	5.93E+06	4.46E+06	4.82E+06		
# spectra	0	5	0	1	1	P00338	L-lactate dehydrogenase A chain
mean intensity	0.00E+00	1.64E+08	0.00E+00	2.59E+06	3.50E+06		

Table 1.5

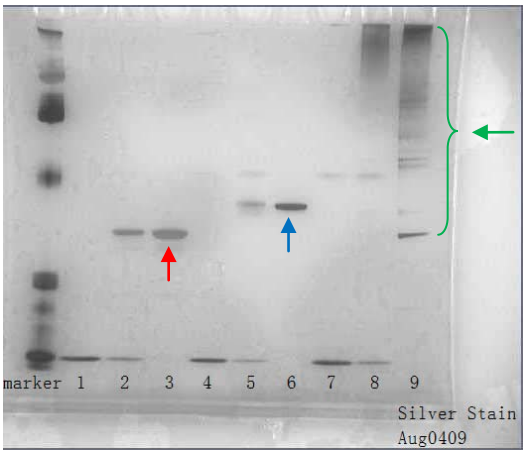
The summary of human proteins identified by MS from on-bead digestion sample of 1-4-KCap pull-down. According to UniProtKB database, these proteins are either actin-binding proteins or glycolytic enzymes.

Protein (from A549)	**Type**
Actin, cytoplasmic 2	
Tubulin alpha-1B chain	Actin-binding
Tubulin beta chain	Actin-binding
Myosin-9	Actin-binding
Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme
Pyruvate kinase isozymes M1/M2	Glycolytic enzyme
L-lactate dehydrogenase A chain	Glycolytic enzyme

actin. We first checked the sequence similarity among these proteins. Although recent research indicates that actin and tubulin may derive from a common ancestral protein, there is no significant sequence similarity between the two proteins in homo species[27, 28]. However, all the proteins identified in the pull-down solution are actin-binding proteins (see section 1.1.3). It is possible that actin and actin-binding proteins formed a complex in the cell lysate and were pulled down by the 1-4-KCap synbody. In order to further reveal the real target of the 1-4-KCap synbody, some binding assays were performed with purified proteins. Figure 1.8 shows the pull-down outcome for purified actin, tubulin and myosin (see section1.2.4 for the sources of the proteins). Each protein was incubated separately with bare beads (as negative control) and beads with 1-4-KCap conjugation. The pull-down products were loaded in an SDS-PAGE gel. Purified proteins

Figure 1.8

IP for 1-4-KCap-Biotin against purified actin, tubulin and myosin. Lane 1, 4, 7, negative control; lane 2, 5, 8, IP against purified protein; lane 3, 6, 9, purified protein only. (100µl of beads, 250nM of synbody, 1µg of actin, 0.2µg of tubulin, 2µg of myosin, Silver Stain; red arrow – actin band, blue arrow – tubulin band, green arrow – myosin bands.)



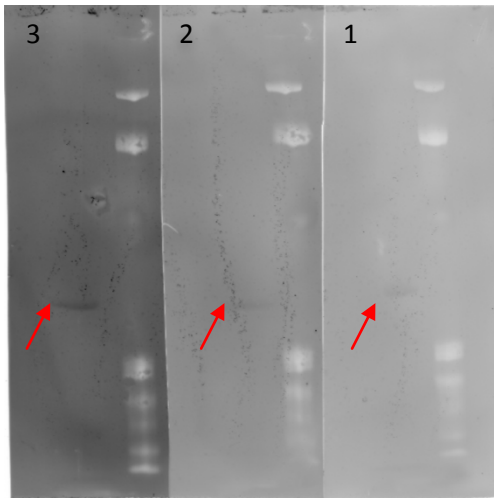
without immunoprecipitation were also directly run in the same gel. The appropriate amount of proteins used in the assays was determined by a previous concentration gradient test of the purified proteins in an SDS-PAGE gel. 1µg of actin and 2µg of myosin were used for the negative control, IP with synbody and direct loading respectively; while for tubulin, only 0.2µg was used for each assay. The only clear bands shown in lane 3 and 6 were actin and tubulin. The positions of the two bands were consistent with the molecular weight of the proteins. Same bands were shown in lane 2 and 5 which indicates the two proteins were pulled down by the synbody. Furthermore, no band appeared in the negative control lanes. The possibility that proteins bound to the

surface of the beads can be excluded. For myosin, however, similar bands were shown in both the negative control and IP with synbody. It is possible that myosin bound to the surface of the beads rather than the synbody. As mentioned previously, although actin and tubulin may come from the same origin, there is no significant sequence similarity between these two proteins. Therefore, the result of the immunoprecipitation indicates that 1-4-KCap synbody might be a multi-specific protein binding reagent.

A Western blot assay was done to further exclude the possibility of binding to the bead surface. The purified actin was run in a gel and transferred to the nitrocellulose membrane. The primary antibody in a regular Western blot assay was replaced by the 1-4-KCap synbody during the detection step. Since the synbody is biotinylated, a streptavidin conjugated Alexa Fluor dye was used for labeling. All the other procedures for the assay were standard for a Western blot (see section 1.2.9). A concentration gradient of both the synbody and the fluorescent dye was tested in the assay (Figure 1.9). The Western blot assay confirms the result that 1-4-KCap synbody binds to actin. Also, this result implies that the synbody has some potential for applications in biological research *in vitro*.

Figure 1.9

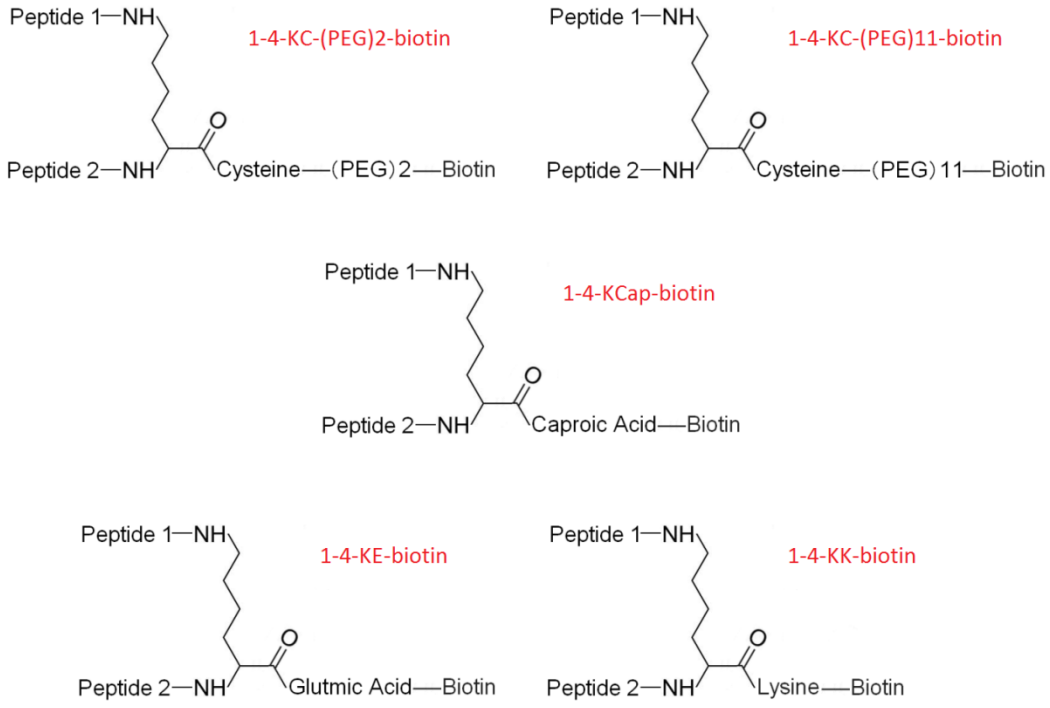
Western blot for actin binding. (Part 1, 10nM of synbody, 10nM of AF488; part 2, 20nM of synbody, 20nM of AF488; part 3, 40nM of synbody, 40nM of AF488; red arrows – actin bands)



Going back to the result shown in Figure 1.6, all the five synbodies contain the same two peptide arms. The only difference among these synbodies is the linker (Figure 1.10). The red arrow in the figure points at the actin bands (~42kDa). The three synbodies with relatively long linker parts show the ability of actin binding while the other two are not able to capture the protein from cell lysate. The interesting phenomenon indicates that the ability of the protein binding may be caused by the property of the linkers rather than the peptide arms. In fact, some research shows evidence that hydroxylated fatty acids derived from arachidonic acid and linoleic acid can bind to the cytosolic actin. The caproic acid, as a type of fatty acid, could be a potential binder of actin[29]. For the1-4-

Figure 1.10

Abbreviated structure of the linkers in synbody.



KC-$(PEG)_2$ and 1-4-KC-$(PEG)_{11}$ synbody, the linkers may also contribute to the actin and tubulin binding. Based on the current result, it is still difficult to explain the interaction clearly. However, further affinity assays focused on the linker may shed light on the mechanism of the binding.

Other proteins, such as the glycolytic enzymes, have not been tested separately for synbody pull-down. Although further study is still necessary for shedding light on improving the specificity of the synbody, the work mentioned previously depicts a reverse process for synbody discovery. The method utilizes mass spectrometry (Figure

1.1). Basically, an unknown synbody is attached to the surface of the magnetic beads, and the potential targets of the synbody are captured in an immunoprecipitation assay. The captured mixture will be identified by the mass spectrometry. Some proteins from the mixture will be selected and used for following affinity binding tests. This type of reverse process is relatively cheap compared with applying synbodies to a protein array. However, for the purpose of rapidly producing human proteome binding reagents, it is still necessary to develop an automated high throughput method for discovering synbodies.

CHAPTER 2

VALIDATION OF PEPTIDE MICROARRAY DATA: A STORY OF LANDING LIGHTS

2.1 Introduction

2.1.1 Immunosignature

The regular process of medical diagnosis is mainly dependent on the patients' clinical symptoms. This type of diagnosis is relatively late and is usually followed by a medical treatment rather than suggestions to aid in prevention. Antibodies are good biomarkers which have been studied a lot in the medical area[29-33]. An immunosignature is an idea which may bring us into an early diagnosis era. Our immune systems produce many antibodies which can specifically bind to different targets, especially, pathogens from the outside world. The composition of all types of antibodies existing in one's blood is related to the individual's health condition. Therefore, if the composition of the complete set of antibodies can be measured, further data analysis may tell us something about people's state of health. If the measurement is applied to a potential infection victim, it may help them to prevent against the appearance of the

symptoms. In theory, a broad range of diseases can be detected pre-symptomatically by this method[34-38].

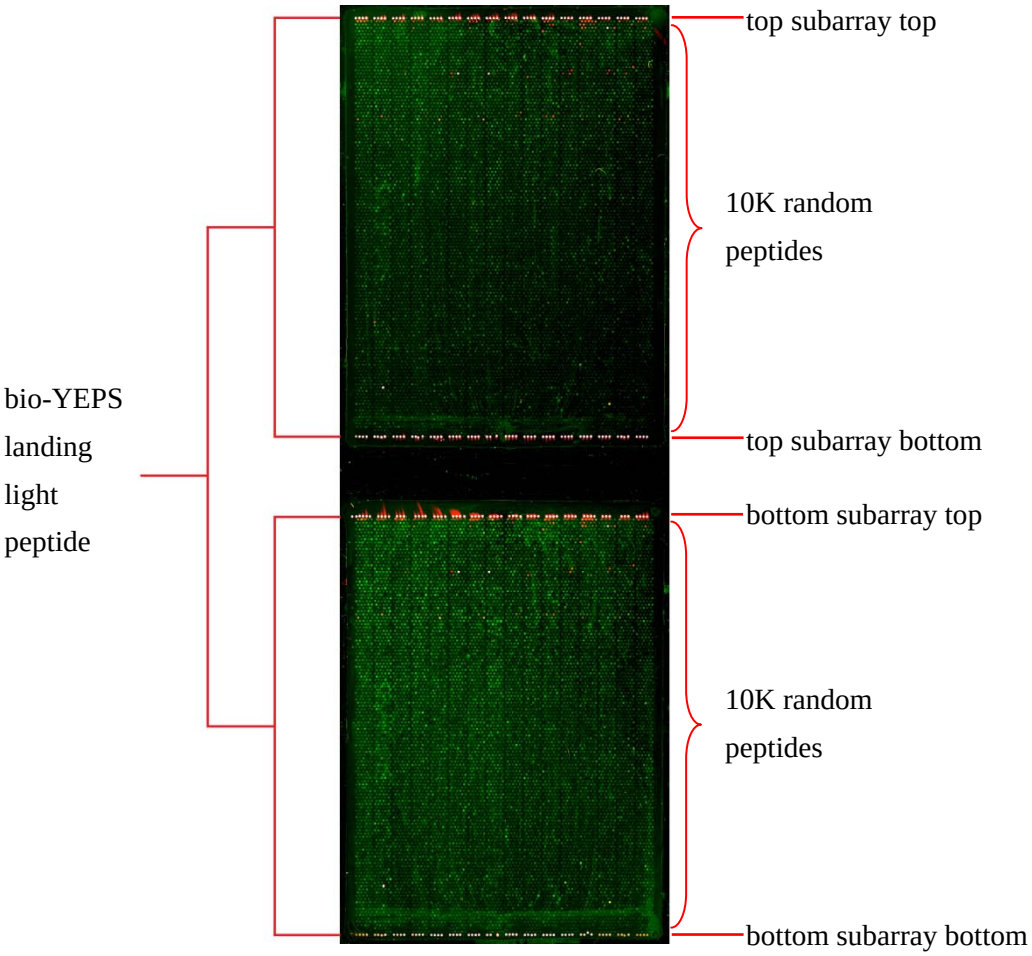
2.1.2 CIM10K Microarray Slide

As mentioned in the synbody project, the randomly synthesized 10K peptide microarray is also a useful tool for acquiring immunosignatures[39]. After spreading one's serum across the 10K peptides imprinted on the slide, the interaction between certain antibodies and their target peptides can be detected with a fluorescent label. The intensity of the fluorescence across the slide provides us with an antibody binding pattern. This pattern is unique and correlates with one's health condition. The sera of people suffering from breast cancer can exhibit similar immunosignature patterns while the sera of flu patients may tell a totally different story. Using the immunosignature technology, we can construct a database of disease profiles. Later on, any blood sample can be immunosignatured and compared with the pre-established database of immunosignatures in order to reveal a potential health issue. Current work done by Joseph Barten Legutki from CIM has proven the feasibility of the new technology[40]. Two distinct immunosignature patterns have been obtained in both mice and human before and after

an influenza vaccination. Further results also prove that unique patterns can be observed by exposure to different pathogens (influenza and tularemia).

The post-experiment data analysis step plays an essential role in the technology of immunosignaturing[40-42]. On each CIM10K slide, peptides are printed in two subarrays which are duplicates (Figure 2.1). In this case, running one slide actually generates two sets of data. Theoretically, the two sets of data from one slide should be identical if both

Figure 2.1
New CIM10K microarray slide.



of the subarrays are treated with the same serum sample. However, variation can be generated during the whole process dealing with the slides, from printing to running. By improving the standard slide operating procedure in the lab, we can minimize the variation caused by slide operation. Nevertheless, the printing step is much more difficult to control. In order to adjust the difference between each two duplicate spots on the same slide, we introduce a landing light peptide which is different from the random peptides. These landing light peptides on the CIM10K slides act as a tool for normalization. The old landing lights used for CIM10K slides are mixed peptides with a variety of concentrations from different sources. They are pre-labeled with a fluorescent dye in prior to printing. The performance of the old landing lights' coefficient of variation is always higher than 10% which is not acceptable for normalization. For the new generation of CIM10K slides, we have started to use a new landing light which only contains one purified peptide with known sequence and concentration. Also, the peptide for the new landing light is biotinylated and it is labeled with fluorescence during slide operation rather than pre-labeled. I modified the peptide with biotin and measured the variation in both test slides and new CIM10K slides.

2.2 Materials and Methods

2.2.1 Slide Activation and Printing

Reagents and Materials

Sulfo-SMCC (Sulfosuccinimidyl-4-N-maleimidomethyl) cyclohexane-1-carboxylate) (bioWORLD # 21810027-1).

Equipment

NanoPrint™ microarrayer printer.

Solution Preparation

Sulfo-SMCC solution: 4.36mg/ml SMCC in PBS with EDTA.

Procedures

For each slide, incubate with 50µl *Sulfo-SMCC solution* in a humidity chamber at room temperature for 1 hour. Wash slides with ultrapure water, three times. Spin to dry slides at 1kRPM (15×g) for 5 minutes. Print slides with the NanoPrint machine.

2.2.2 Landing Light Test I

Reagents and Materials

Streptavidin, Alexa Fluor® 555 conjugate (Invitrogen™ # S32355); Streptavidin, Alexa Fluor® 647 conjugate (Invitrogen™ # S32357).

Equipment

ProScanArray (PerkinElmer®).

Solution Preparation

Prewash Solution: 33% isopropanol, 7.3% acetonitrile, 0.55% TFA in ultrapure water; *Blocking Buffer:* 3% BSA, 0.05% Tween-20, and 0.134mg/ml β-Mercaptohexanol in 1X PBS; *Incubation Buffer:* 3% BSA and 0.05% Tween-20 in 1X PBS.

Procedures

Wash the printed slides with *Prewash Solution* for 5 minutes. Wash with ultrapure water, three times. Spin to dry at 700RPM for 3 minutes. Block the surface of slides with 700µl of *Blocking Buffer* in a humidity chamber at room temperature for 1 hour. Wash slides with TBST; then wash with ultrapure water, twice. Spin to dry at 700RPM for 5 minutes. Incubate slides with 5nM streptavidin conjugated Alexa Fluor® dye in *Incubation Buffer* (250µl in each block for a 2-up gasket) at 37°C for 1 hour, using Agilent chamber and gasket slide. Wash slides with TBST, 3 times; then wash with ultrapure water, 3 times. Spin to dry at 700RPM for 5 minutes. Scan slides by ProScanArray.

2.2.3 Landing Light Test II (CIM10K)

Reagents and Materials

Streptavidin, Alexa Fluor® 555 conjugate (Invitrogen™ # S32355); Streptavidin, Alexa Fluor® 647 conjugate (Invitrogen™ # S32357).

Equipment

HS 4800™ Pro (Tecan®); ProScanArray (PerkinElmer®).

Solution Preparation

Prewash Solution: 33% isopropanol, 7.3% acetonitrile, 0.55% TFA in ultrapure water; *Blocking Buffer:* 3% BSA, 0.05% Tween-20, and 0.134mg/ml β-Mercaptohexanol in 1X PBS; *Incubation Buffer:* 3% BSA and 0.05% Tween-20 in 1X PBS.

Procedures

The immunosignature assay was performed by Rebecca Halperin with HS 4800™ Pro (Tecan®). After blocking the surface of the slides, human serum samples were loaded onto the slides and the specific binding between serum antibody and the 10K random peptides was identified by anti-human secondary antibody. The streptavidin conjugated Alexa Fluor dye 555/647 was used for fluorescent labeling.

2.3 Results and Discussions

2.3.1 Biotinylated Peptide Preparation

The peptide used for the landing lights on the new CIM10K slides was previously synthesized and purified. It was stored in the -20°C freezer in solid form. The sequence of the peptide is YEPSDAFKITRFHQQSSGSC (YEPS peptide). The sulfhydryl group of the C terminal Cysteine was used to react with sulfo-SMCC and immobilize the peptide onto the slides. The YEPS peptide was biotinylated by NHS-PEG_4-biotin which targets free amino groups. Two amino groups are available in the peptide, one from the N terminal Tyrosine and the other is from the side group of Lysine. After the biotinylation, the excess biotin was removed using a MICROCON® Centrifugal Filter Devices Ultracel YM-3 filter (3kDa). In theory, only the peptide with two biotins attached (bio-YEPS peptide) (~3402Da) would be retained above the filter, whereas the NHS-PEG_4-Biotin (~588Da), the peptide with no biotin (~2456Da) and one biotin (~2929Da) attached would pass through the filter. The supernatant was purified using HPLC (Figure 2.2, HPLC performed by Zbigniew Cichacaz from CIM) and the solution collected at 21.763min was sent for MALDI measurement (Figure 2.3). The difference of the molecular weight before and after biotinylation (~959Da) coincided with the theoretical value for two biotin additions (~946Da). Two sets of the bio-YEPS peptide (0710 & 0810) were made. 0710 was only used for the test while 0810 was applied to both the test slides

and the new CIM10K slides. All the data shown here was generated by the second set of bio-YEPS peptide (0810). Similar results were obtained from the 0710 set (data not shown).

Figure 2.2

The chromatogram of the supernatant collected from biotinylation mixture.

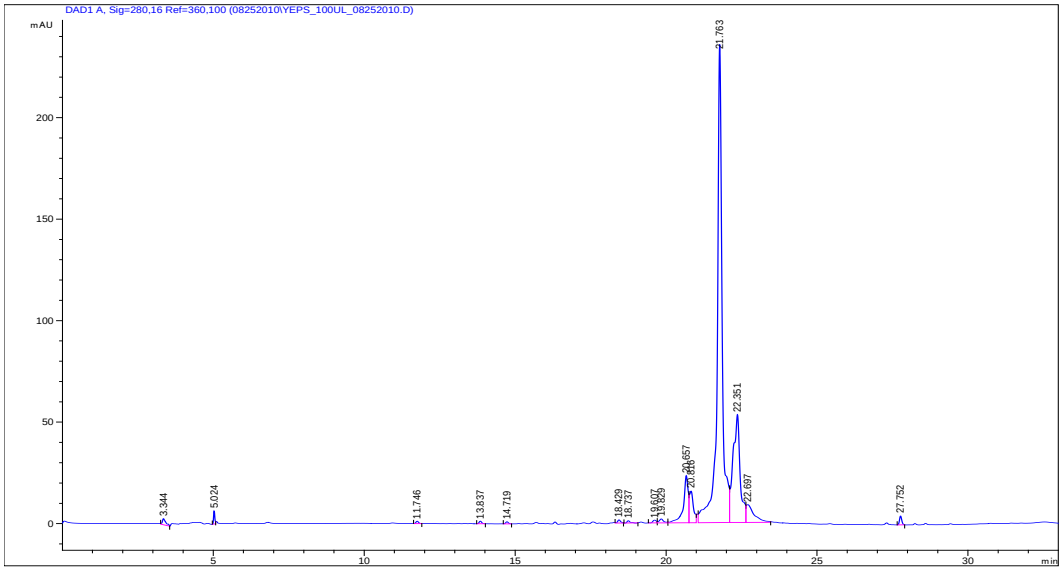
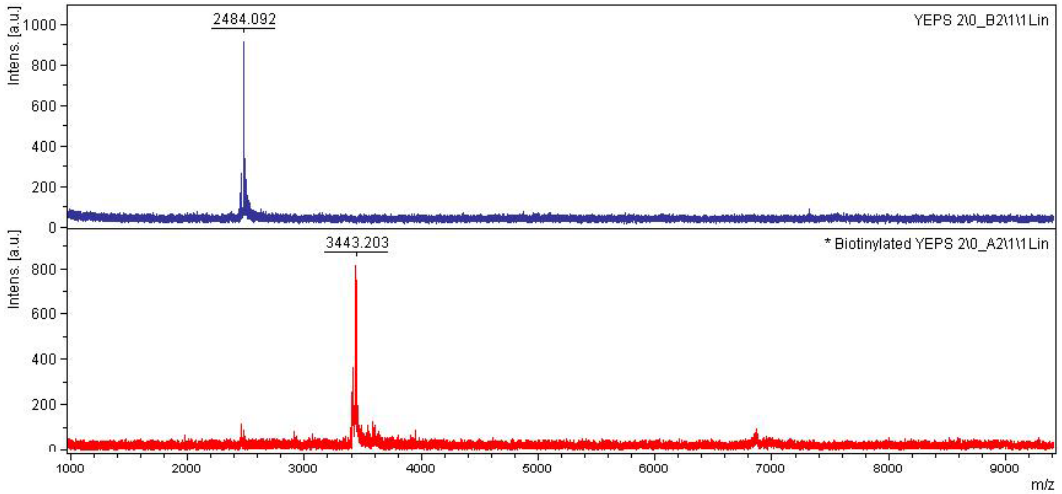


Figure 2.3

Mass spectrum of YEPS peptide (top) and bio-YEPS peptide (bottom).

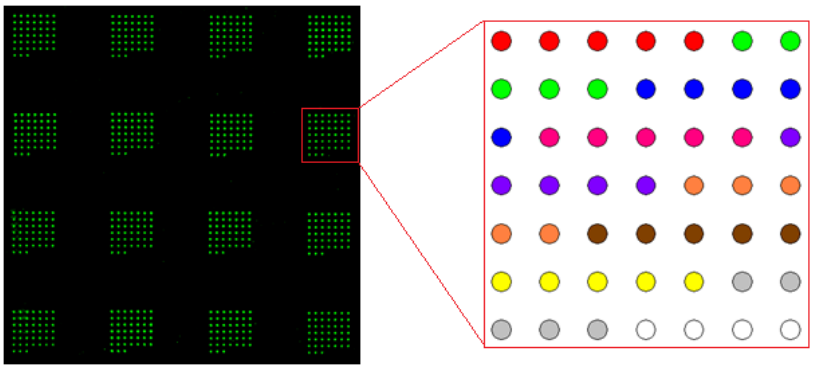


2.3.2 Improvement of the Landing Lights

A series of dilutions (0.75mg/ml, 0.5mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.075 mg/ml, 0.05mg/ml, 0.025 mg/ml, and 0.0125 mg/ml) of the bio-YEPS peptide was prepared based on the concentration measurement obtained with the NanoDrop instrument. The concentration series of bio-YEPS peptide together with the old landing light peptide, which is pre-labeled with Alexa Fluor dye, was printed on the test slides. The glass surface of the slides was originally covered with aminosilane. Before printing, the slides were activated by a maleimide linked sulfo-SMCC which could react with the sulfhydryl group of the peptide[43]. Two sets of test slides (0710 & 0810, each set contains 10 slides) were printed prior to the new CIM10K slides. The set number of the slides coincided with the set number of the bio-YEPS peptide which was used for printing. The old landing light on both sets of slides was from the same source. Also, both sets of slides shared the same printing pattern. A top subarray and a bottom subarray which both contained 16 identical blocks of spots that were printed on each slide. The printing pattern for each block is shown in Figure 2.4. Sixteen pins were used for printing. The spots in each block were printed by the same pin.

Figure 2.4

The printing pattern of a subarray and a block. Red - 0.75mg/ml; green - 0.5mg/ml; blue - 0.25mg/ml; pink - 0.125mg/ml; purple - 0.075mg/ml; orange - 0.05mg/ml; brown - 0.025mg/ml; yellow - 0.0125mg/ml; gray – regular landing lights; white - empty.



After the prewash, blocking and incubation with fluorescent dye (5nM in *Incubation Buffer*), the slides were sent to ProScanArray (PerkinElmer®) for scanning. In each set of the slides, we added Streptavidin, Alexa Fluor® 555 conjugate (AF555) to five slides and Streptavidin, Alexa Fluor® 647 conjugate (AF647) to the other five. All of the other operating procedures were the same. We initially tried to scan the slides with 70% PMT gain. Under this setting, all the spots on the slides blew out which means that the intensity of the spots reached the maximum detectable value and further data analysis would be meaningless. 40% PMT gain was finally chosen for scanning after several tries with different settings. All the data shown below was generated with 40% PMT gain.

The scan pictures of the slides were processed by GenePix Pro 6.0 (Axon Instruments). The gal file generated by John Lainson from CIM was applied to both sets

of slides for alignment. After the alignment, the results were saved as GPR files which can be opened with MS EXCEL. The data corresponding to the F555/F647 median intensity was used for analysis. For every five spots with the same concentration from the same block, we calculated the standard deviation (σ) and the coefficient of variation (CV) based on the median intensity. Then, we averaged the CV among each of the 5 slides labeled with the same dye. This is called the spot-to-spot CV. The spot-to-spot CV data is shown in Figure 2.5 and Figure 2.6. Every sixteen data spots (top or bottom) located in the same interval (as marked in the figure) came from the same concentration. Different data spots in the same interval came from the spots printed by different pins. The location of the data spots repeated the same pattern among intervals which indicated that the quality of the pins may be affecting the CV of the landing lights significantly. Although a few spots showed high CV, either the value or the increasing trend of most of the data from the bottom subarray is similar with the data from the top one. From 0.75mg/ml to 0.125mg/ml, the CVs remained low (under 5%). From 0.075mg/ml to 0.0125mg/ml, the CVs went up as the concentration went down. The data spots from the old landing lights showed more variation than the data spots from the new landing lights. We also averaged the data from old and new landing lights in different concentrations, shown in Figure 2.7

and Figure 2.8. All the data shown here is based on the slide set 0810. Similar results were obtained from slide set 0710 (data not shown).

Figure 2.5
Average CV among 5 slides labeled with AF555 from set 0810. Blue, data from top subarray. Red, data from bottom subarray.

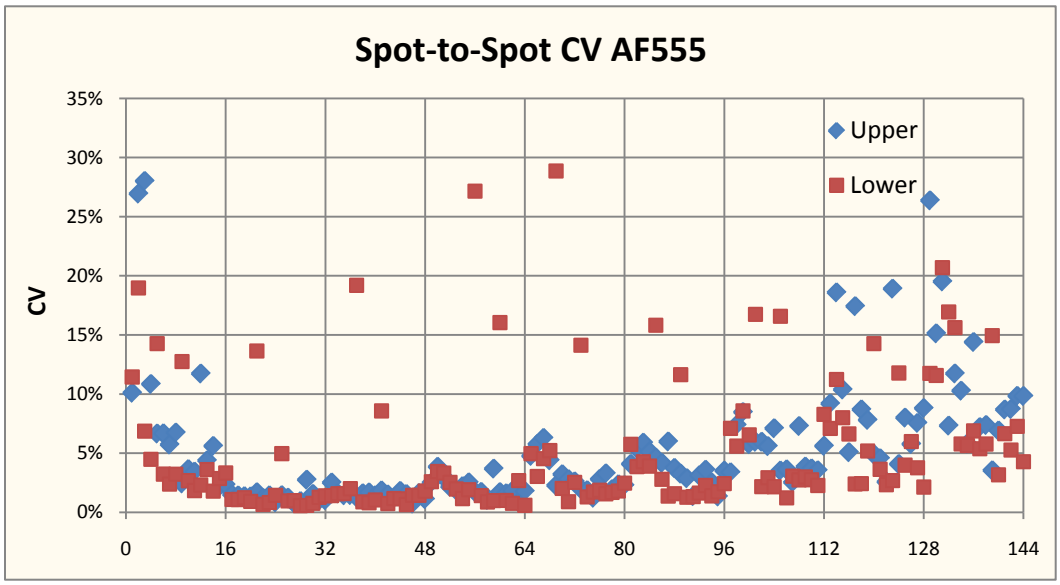


Figure 2.6 Average CV among 5 slides labeled with AF647 from set 0810. Blue, data from top subarray. Red, data from bottom subarray.

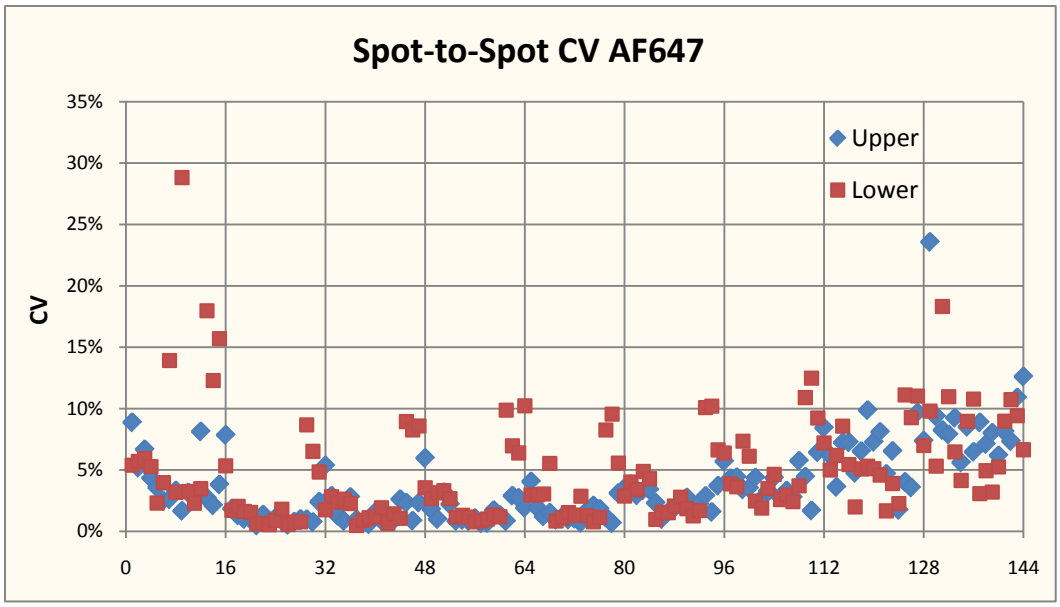


Figure 2.7

Average of spot-to-spot CV, slides labeled with AF555 from set 0810. Blue, data from the top subarray. Red, data from the bottom subarray. (RLL - regular landing lights)

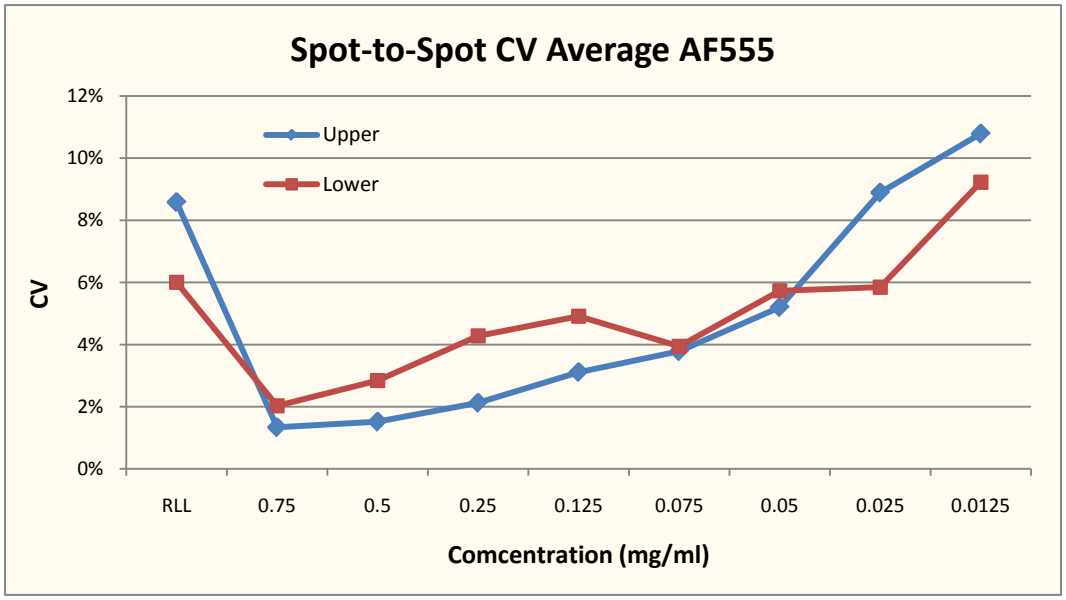
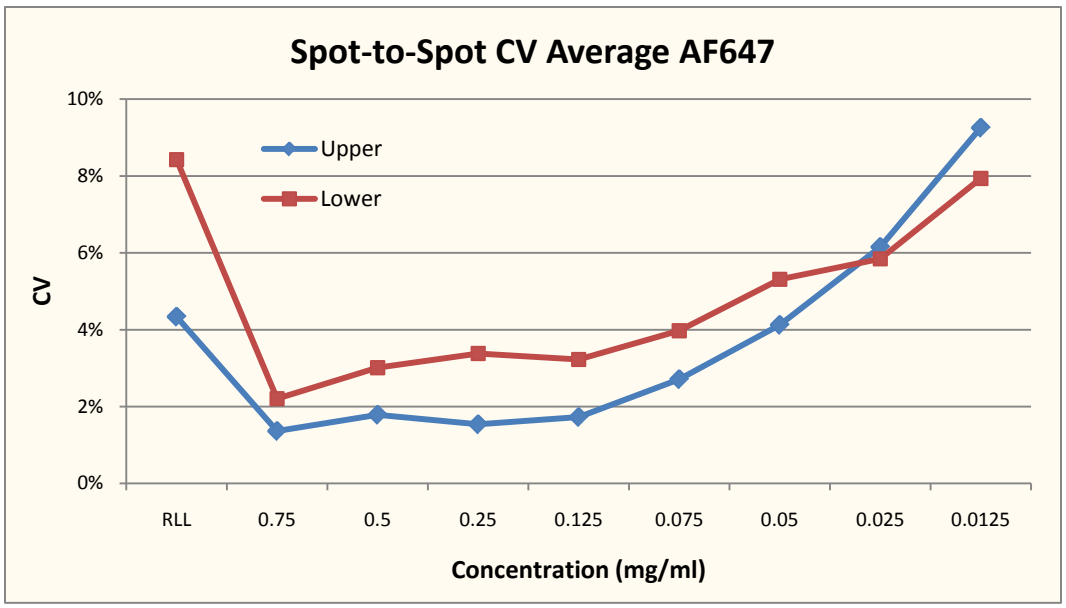


Figure 2.8

Average of spot-to-spot CV, slides labeled with AF647 from set 0810. Blue, data from the top subarray. Red, data from the bottom subarray. (RLL - regular landing lights)



The regular landing lights are mixed peptides with various concentrations. They are conjugated with Alexa Fluor dye before printing. The concentration of the mixture can be one reason for the high variation of the intensity. Also, the prewash step with TFA after printing may wash off the previously labeled dye. Both of these two factors which significantly affect the variation were improved with the new landing lights. The bio-YEPS peptide used as a new landing light is purified and the concentration of the peptide printed on the slides was known. Both Alexa Fluor dyes used for lighting up the landing light and the 10K peptides are applied to the slides at the same time. Therefore, both dyes undergo the same incubation and wash steps. The data shown in the test of bio-YEPS peptide indicates an improvement of intensity variation for higher concentrations (>0.1mg/ml) of the new landing light. The improvement is shown as a fold change in Table 2.1. Also, no significant difference is shown between the two sets of slides using

Table 2.1
Improvement of the intensity variation.

Dye	Regular landing light CV	New landing light CV	Fold change
AF555	7.293%	2.769% (average 0.125~0.75mg/ml)	2.62
		1.682% (0.75mg/ml)	4.34
AF647	6.382%	2.279% (average 0.125~0.75mg/ml)	2.80
		1.785% (0.75mg/ml)	3.58

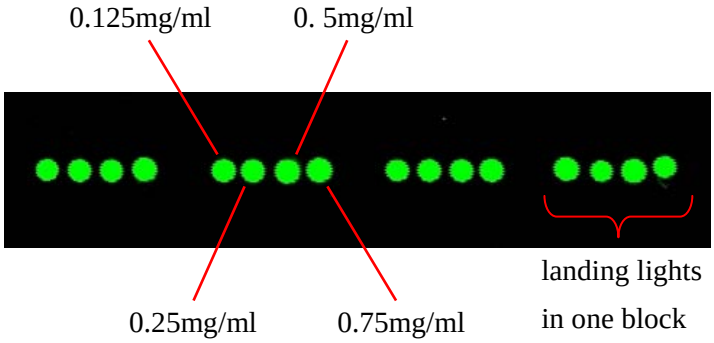
two different dyes. Finally, the concentration series, 0.75mg/ml, 0.5mg/ml, 0.25mg/ml and 0.125mg/ml was chosen for the new CIM10K slides fabrication.

2.3.3 Landing Lights on New CIM10K Slides

New CIM10K microarray slides were printed with bio-YEPS peptide by Applied Microarrays, Inc (AMI). The surface condition of the slides printed by AMI was the same as the condition used for the test slides – with sulfo-SMCC activated aminosilane. The positions of the landing lights on the slides are shown in Figure 2.1. All the slides used here were run by Rebecca Halperin for immunosignature tests. The slides were operated by HS 4800™ Pro (Tecan) and the landing lights were labeled by 5nM streptavidin conjugated Alexa Fluor® 555/647. The original scan data was created with a higher PMT setting (Agilent's DNA Microarray Scanner with SureScan High-Resolution Technology). The median intensity of most landing light spots reached the maximum detectable value. In this case, the CV among the intensity of the spots is invalid and is not eligible for the following analysis. In order to compare with the previous landing light test results, the slides were rescanned with 40% PMT by ProScanArray (PerkinElmer®). During the data analysis procedure, all the landing light spots were divided into four groups: TST - top subarray top, TSB - top subarray bottom, BST - bottom subarray top, and BSB - bottom

subarray bottom. By directly checking the scan images, a difference of the intensity among the four groups of landing lights was shown. Therefore, the data of distinct groups of landing lights was analyzed separately. The printing pattern of each group of landing lights is the same and shown in Figure 2.9. We have four landing light spots with distinct concentrations printed in each block both at the top and bottom. The concentration increases from left to right. During the data analysis step, spot-to-spot comparison was based on the variation among spots with same concentration in the same group on one

Figure 2.9
Printing pattern of landing lights on new CIM10K microarray.



slide (e.g. all 16 spots with 0.25mg/ml in group TST on slide 101). Because the derived data was significantly affected by one missing spot (caused by printing) in TSB and BSB with 0.5mg/ml, the average intensity and CV were calculated by the other 15 spots in these groups.

Figure 2.10 shows the average intensity of the spots in the same group with the same concentration. It is obvious that landing lights located at the top in each subarray have a higher intensity compared with the spots at the bottom. Also, the average intensity of the top subarray is higher than the bottom one. The error bars in Figure 2.10 are based on the variation of average intensity among all slides. The average spot-to-spot CV for different groups is shown in Figure 2.11. The data of TST, TSB and BST is very similar while BSB has a surprisingly high CV. By checking the data of individual slides, two slides with significantly high spot-to-spot CV were found. The original scan images explained the reason for the high CV. The BSB landing light spots on one slide were cut

Figure 2.10
Average intensity comparison for different groups and concentrations.

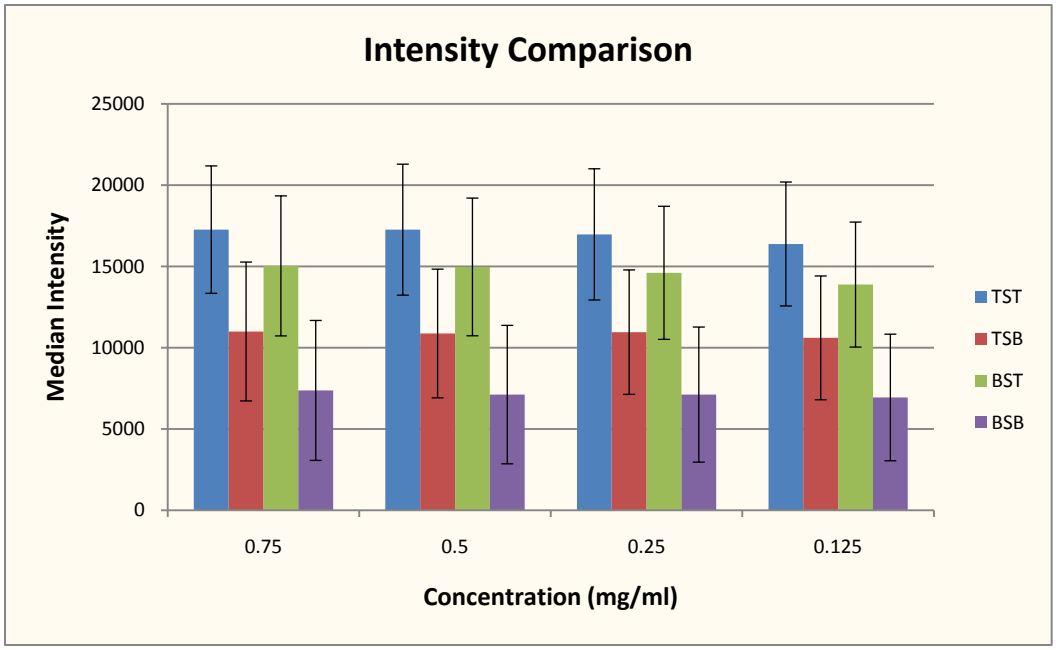
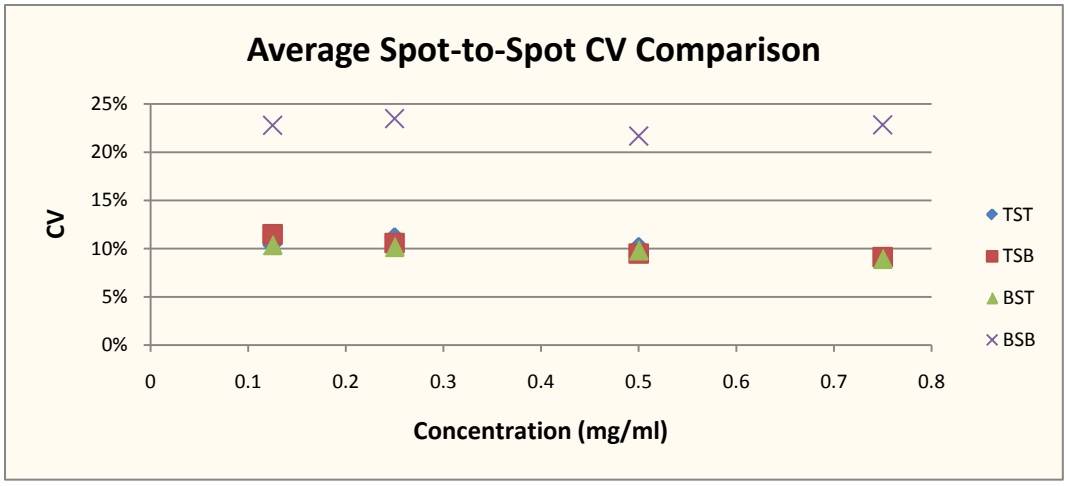


Figure 2.11

Average spot-to-spot CV comparison for different groups and concentrations.

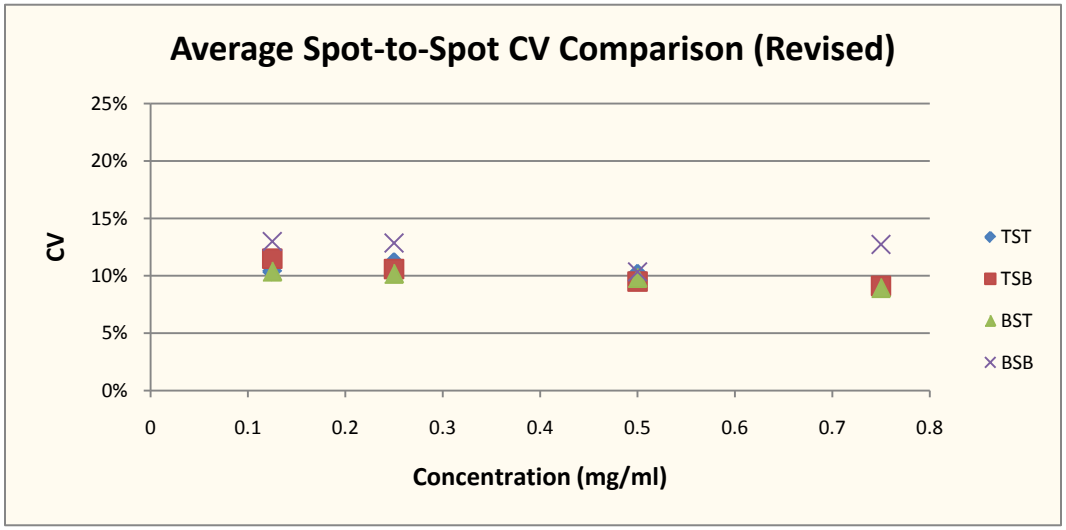


by the rubber frame of the gasket during the incubation which resulted in a CV around 150%. On the other slide, the BSB landing lights were very close to the frame of the gasket and exhibited a very low intensity. After removing the data of the two questionable slides, a revised graph of average spot-to-spot CV comparison was made (Figure 2.12).

Although the CVs of BSB are lower than the data shown previously, they are still relatively high compared with the other groups. This unexpected result pointed out a potential problem with the printing process. As mentioned previously, two slides had some issue with the BSB landing lights. In fact, this problem happened to all slides which used either the Tecan or Agilent gasket during incubation. Scan images from all sets of the new CIM10K slides were checked. The location of the spots in the top subarray

Figure 2.12

Average spot-to-spot CV comparison (revised) for different groups and concentrations.



matched the rubber frame of the gasket while the location of the bottom one was shifted a little so that the BSB landing lights were close to the edge of the frame. One outcome of the shift is that the landing light spots are “cut”. Also, even if the spots are not cut, the incubation solution may not flow smoothly near the edge of the frame. This is another possible reason why the variation of the intensity of BSB landing lights was higher than the others. Therefore, the BSB landing lights are not suitable to be used for normalization.

Apart from the BSB landing light issue, the overall CV for the bio-YEPS peptide on the new CIM10K slides (around 10%, Figure 2.12) is not as good as the one shown in the early test (under 5%, Figure 2.7 & 2.8). Most of the CV data for individual CIM10K slides are low (around 5%). The higher overall CV is caused by some slides which

exhibit a greater variation (around 30%). It seems the property of the bio-YEPS peptide is reliable for working as a landing light when printed by AMI. However, after the incubations with various types of sera and secondary antibodies, the variation of the intensity increases.

Several problems still need to be solved before the landing lights can be used for normalization in immunosignature assays. The intensity of the landing light spots reaches the maximum value after scanning with normal settings. In this case, the data is not eligible for normalization. The new CIM10K slides from Rebecca Halperin were rescanned with 40% PMT for collecting meaningful data. However, under this PMT setting, the signals of the 10K random peptides are too low for analysis. In this situation, it is impossible to have one's cake and eat it too. Although the intensity can be lowered by reducing the concentration of the landing lights, the variation of the landing light intensity increases as the concentration is decreased (see Figure 2.7, 2.8 and 2.12). Reducing the concentration of the streptavidin dye applied to the slides may not help, either. The intensity of the landing lights still reached the maximum when 0.1nM of the dye was applied to the epoxysilane slides (for the DARPA project). There is no significant improvement compared with 5nM of the dye on aminosilane slides (for the

Immunosignature project). An alternative strategy is to select peptides from the 10K random sequences for backup landing lights[42]. Three peptides with consistently high intensity across the slides were selected. The sequences of the peptides are CSGSPIVLLSMPQPLETGRQ, CSGNWLRHFAERSFSLNGIH, and CSGQWVRTQLATFLLTTASE. The reason why these peptides had such high intensity after incubation is not clear. Although these peptides don't share the amino acid sequence with some known streptavidin binding peptides[5, 44], it is still possible that they are able to capture streptavidin conjugated Alexa Fluor dye in the incubation solution. This hypothesis can be tested by several types of binding assays. Nevertheless, with the normal PMT setting, these three peptides also had a high intensity close to the maximum value, which makes these signals unsuitable for normalization.

Based on the results of the landing lights, a possible improvement for more reliable data can be created by separating the labeling systems for the landing lights and the 10K random peptides. During the last step of incubation in an immunosignature assay, the streptavidin dye is used for labeling both the landing lights and the secondary antibodies which bind to antibodies captured by 10K peptides from the sera. The concentration of the dye applied to both spots is the same. If the biotinylated secondary

antibodies are replaced by antibodies with a pre-labeled dye, the streptavidin dye will only react with the landing lights. Although a low concentration of the dye was not able to prevent the intensity from reaching the maximum on epoxysilane slides, we haven't test low concentrations on the aminosilane slides. Also, lowering the concentration may have some advantages which allow the variation to be controlled. Therefore, separating the labeling systems may make it possible to improving the quality of data for normalization.

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