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Ligand-directed profiling of organelles with internalizing phage libraries

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Abstract

Phage display is a resourceful tool to, in an unbiased manner, discover and characterize functional protein-protein interactions, to create vaccines, and to engineer peptides, antibodies, and other proteins as targeted diagnostic and/or therapeutic agents. Recently, our group has developed a new class of internalizing phage (iPhage) for ligand-directed targeting of organelles and/or to identify molecular pathways within live cells. This unique technology is suitable for applications ranging from fundamental cell biology to drug development. Here we describe the method for generating and screening the iPhage display system, and explain how to select and validate candidate internalizing homing peptide.

INTRODUCTION

Biochemical and genetic techniques such as affinity capture complex purification and yeast two-hybrid are commonly used for protein interaction studies, including the discovery of

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intracellular receptors. However, such approaches are financially and labor-intensive procedures, and more importantly, given their artificial nature, i.e. *in vitro* or fusion proteins, can lead to the identification of artifactual, and/or miss physiologically-relevant interactions partners. As a consequence, many candidate protein-protein interactions cannot be functionally validated. Within this context, phage display is an alternative and versatile method for deciphering the molecular diversity of peptide binding specificity to isolated proteins, purified antibodies, cell surfaces, intracellular/cyto-domains, and blood vessels *in vivo*. Recently, we have developed a new technology for screening internalizing phage (iPhage) vectors and libraries with a ligand/receptor-independent mechanism to penetrate eukaryotic cells. This provides a novel platform for the discovery of intracellular interactions in live cells.

In this unit, we explain the manipulation and preparation of the f88-4 and fUSE5 phage display vectors as well as the design, cloning, construction, and production of iPhage-based vectors and libraries (Basic Protocol I). Additionally, critical steps required to select, identify, and validate candidate internalizing homing peptide motifs and their corresponding organelle receptors and native ligands are thoroughly detailed (Basic Protocol II).

BASIC PROTOCOL I

PREPARATION OF IPHAGE LIBRARY

Phage display, i.e., the display of peptides on the surface of filamentous phage by genetic engineering, was first introduced in 1985 by George Smith. Since then it has become a simple and extremely powerful tool widely used to rapidly and efficiently characterize protein-protein interactions of a substantial number of candidates. Phage display has been exploited to create vaccines, and to engineer peptides, antibodies and other proteins as both diagnostic tools and targeted therapeutic agents (Smith & Petrenko, 1997; Bratkovic, 2010; Molek et al, 2011; Thie et al, 2008). Our group has been using phage display technology to identify receptor-ligand interactions *in vitro* and *in vivo*, which has helped to extend its potential for drug development (Cardo-Vila et al., 2008; Giordano et al., 2010; Barnhart et al., 2011). This combinatorial selection methodology has proven versatile also when applied to cells *in vitro*; indeed, the biological diversity of the cell surface can be probed even when cells have been removed from their usual tissue architecture (Kolonin et al., 2006; Giordano et al., 2008). More recently we have developed a new technology for screening internalizing phage (iPhage) vectors to study metabolic pathways and identify intracellular and organelle receptors (Rangel et al., 2012). This new class of bacteriophage-based reagents integrates the recombinant penetratin (pen) as a fusion protein on a recombinant major phage coat protein (rpVIII), and thereby enables receptor-independent phage particle entry into, and intracellular distribution within, mammalian cells. Moreover, a random peptide library displayed on the minor coat protein (pIII) allows intracellular library selection.

The construction of phage random peptide libraries is based on cloning DNA fragments encoding peptide sequences into the phage genome fused to the pIII coat protein gene. Incorporation and expression of the gene fusion product results in the presentation of the peptide on the phage surface, where it can interact with and bind to a potential target. A phage library can consist of up to 10^9 unique phage clones, each displaying a different

peptide. The size of the peptide insert, as well as its expression orientation (linear or cyclic), are two parameters that can be adjusted to best fit the purpose of the screen. The success of the screening is integrally dependent on how well the library is constructed. If the iPhage constructs are properly assembled, iPhage library titers of $1\text{--}5 \times 10^{10}$ Transducing Units (TU)/ml are routinely obtained and are consistent with the titers generated with the parental phage. Moreover, generation of iPhage particles is often abundant and non-toxic to the host bacteria K91/kan *E. coli*. Functional assays to evaluate either correct display of penetratin and/or the capacity of targeted iPhage to home to different intracellular compartments, such as mitochondria (exemplified in this protocol), can be performed by infection of host *E. coli* and by the iPhage internalization assay, respectively (Rangel et al., 2012).

The phage vector commonly used for the construction of random peptide libraries is the fUSE5 plasmid. The fUSE5 vector was engineered to be non-infective by disrupting the gene III reading frame with a 14-bp “stuffer” (Smith & Scott, 1993). Infectivity is restored only when the “stuffer” sequence is replaced with an in-frame insertion. Removal of the fUSE5 “stuffer” sequence within gene III is achieved by digestion with the restriction enzyme *Sfi*I. This process leaves two overhanging sites incompatible with each other thereby allowing the unidirectional cloning of the DNA insert (Smith & Scott, 1993). Here, we describe the procedures outlining the steps for generating, isolating, and purifying iPhage vectors (Basic Protocol I) along with the generation, library screening, clone selection, and organelle receptor validation by iPhage technology (Basic Protocol II).

Materials—fUSE5 and f88/4 phage plasmids are available upon request from the University of Missouri (<http://www.biosci.missouri.edu/smithgp/PhageDisplayWebsite/PhageDisplayWebsiteIndex.html>)

Electrocompetent DH5 α competent cells (Life Technologies)

0.5 ml microfuge tubes

0.1 cm electroporation cuvette (Bio-Rad)

Pasteur pipettes

Super Optimal Broth with Catabolite repression (SOC, APPENDIX).

Luria-Bertani (LB) medium and agar plates (APPENDIX).

Tetracycline stock (APPENDIX)

QIAGEN Plasmid Maxi Kit

10 mM Tris-HCl, pH 8.0

Cesium chloride (CsCl, Fisher Scientific)

Ethidium bromide solution (EtBr, 10 mg/ml; Bio-Rad)

Ultracentrifuge tubes (Thermo Fisher Scientific)

18g needle (Thermo Fisher Scientific)

1 ml syringe (Thermo Fisher Scientific)

15 ml screw-cap polypropylene tubes

Isoamyl alcohol (Fisher Scientific)

3M Sodium acetate (SIGMA)

Ethanol (Fisher Scientific)

70% Ethanol

Penetratin Forward 5'-
CACAAAGCTTTGCCAACGTCCCTCGACAGATAAAGATTTGGTTCCAAAACGGCGC
ATGAAGTGGAAGAAGCCTGCAGCACA-3'

Penetratin Reverse 5'-
TGTGCTGCAGGCTTCTTCCACTTCATGCGCCGGTTTTGGAACCAAATCTTTATCT
GTCGAGGGACGTTGGCAAAGCTTGTG-3'

5000 units of *Hind*III endonuclease (10U/μl, Fermentas)

3000 units of *Pst*I endonuclease (10U/μl, Fermentas)

QIAquick Gel Extraction Kit (QIAGEN)

0.8 and 2% agarose gels

Quanti-Marker 100 bp (Bioexpress)

QIAprep Spin Miniprep Kit (QIAGEN)

30% glycerol-LB

MC1061 *E. coli* competent cells. The MC1061 *E. coli* strain can be obtained from the University of Missouri (Dr. George Smith)

SOB Media (APPENDIX)

Streptomycin stock (APPENDIX)

500 units of T4 DNA ligase (1U/μl, Life Technologies)

f88/4 forward 5' - GCTCCTTTCGCTTTCTTCCCTTCC-3'

f88/4 reverse 5' - TCAGGGGAGTAAACAGGAGACAAG-3'

1500 units of *Xba*I endonuclease (10U/μl, Fermentas)

4000 units of *Bam*HI endonuclease (10U/μl, Fermentas)

Ultraviolet transilluminator

10% glycerol

1000 units of *Sfi*I endonuclease (10U/μl, Fermentas)

Quanti-Marker 1Kb (Bioexpress)

Library template 5'-

CACTCGGCCGACGGGGCTNNKNNKNNKNNKTATNNKNNKNNKNNKGGGGCCG
CTGGGGCCGAA-3'

10 mM dNTPs (Life Technologies)

GoTaq DNA polymerase (5U/μl; Promega) [includes 5X buffer, MgCl₂ solution]

Dimethyl sulfoxide (DMSO, SIGMA)

Library sense primer 5'-CACTCGGCCGACG-3'

Library antisense primer 5'-TTCGGCCCCAGCGGC-3'

QIAquick nucleotide removal Kit (QIAGEN)

4% Agarose gels

2000 units of *Bgl*II endonuclease (10U/μl, Fermentas)

High Throughput Electroporation plates (BTX Harvard Apparatus)

Polyethylene glycol (PEG)-sodium chloride (NaCl) (16.7% 13.3M stock; APPENDIX)

Phosphate buffered saline (PBS), pH 7.4

K91/kan *E. coli*. The bacteria strain is obtained from the University of Missouri (Dr. George Smith).

Terrific broth (TB, APPENDIX)

Kanamycin stock (APPENDIX)

Generating an internalizing phage peptide library—In this section, we describe purification of the phage vector from the single strand DNA, the cloning strategy used to produce the iPhage vector, and production of the random peptide library (Figure 1).

Propagating f88-4 and fUSE5 phage plasmids—

1. Electroporate 10 ng of plasmid (i.e., f88-4, fUSE5) in 20 μl of DH5α *E. coli* (Invitrogen). Thaw the bacteria on ice and place in chilled 0.5 ml microfuge

tubes. Mix the plasmid and bacteria and transfer into a 0.1 cm electroporation cuvette. Electroporate using the following conditions: 1.8 kV, 200 ohms, 25 μ F (Bio-Rad). *Avoid introducing air bubbles into the cell/DNA mixture to prevent arcing during electroporation.*

2. Using a Pasteur pipette transfer the electroporated bacteria to 1 ml of SOC medium and incubate at 37°C for 1 hr.
3. Plate serial dilutions (1:10, 1:100, 1:1000) on LB-tetracycline (tet) plates and incubate overnight at 37°C.

Note that E. coli transformed with phage vectors grow more slowly and may require slightly longer incubation times to obtain visible colonies. Select the best colony plate, seal with parafilm, and store at 4°C.

Plasmid purification by cesium chloride (CsCl) gradient—CsCl gradient is the most reliable method to obtain highly purified plasmid. Ultracentrifugation forces establish a density gradient that allows the separation of proteins, RNA, and single strand from double strand DNA. This method of purification has no commercial substitution and is the best system to obtain plasmid preparations for iPhage cloning and library construction.

4. Prepare a seed culture from a single DH5 α *E.coli* colony (method above) in 5 ml of LB-tet (40 μ g/ml) media under agitation (225 rpm) for 8 hr at 37°C.
5. Add the starter culture to 500 ml of LB-tet media and shake overnight at 37°C. Use a 2 L flask to ensure sufficient air for the overnight culture.
6. Centrifuge the culture at 6,000g for 15 min at 4°C, and purify using the maxi-prep plasmid purification kit (QIAGEN).

To increase the plasmid yield, warm the elution buffer to 50°C.

7. Prepare a dilution of the DNA solution in 10 mM Tris-HCl, pH 8.0. Mix well, and measure the absorbance of the dilution at 260 nm (A_{260}) in a spectrophotometer blanked against 10 mM Tris-HCl, pH 7.5. Calculate the concentration of DNA using the following formula:

$$DNA(\mu g/\mu l) = \frac{OD_{260} \times 50\mu g/ml \times Dilution\ Factor}{1,000} \quad \text{Eqn. 1}$$

8. Measure the plasmid DNA volume, and add 1.1 g of CsCl₂ per ml of plasmid solution. Dissolve the CsCl by mixing gently, and prepare 10 mM Tris-HCl, pH-8.0/CsCl solution for a balance tube. Add 70 μ l of ethidium bromide (EtBr; 10 mg/ml) per ml. The following steps are done under low light intensity to avoid DNA mutations due to the exposure to EtBr.

EtBr is a potent mutagen. It may be fatal if inhaled and is harmful if swallowed or absorbed through skin. Causes irritation to eyes, respiratory tract and skin. May cause heritable genetic damage. Wear gloves and safety glasses. It must be

handled with extreme caution and decontaminated on activated charcoal or amberlite ion exchange resins prior to disposal.

9. Remove any insoluble particles of the EtBr present in the CsCl-DNA solution by spinning the tube at 1,000g for 10 min at room temperature. Transfer the clear supernatant to an ultracentrifuge tube (Thermo Fisher Scientific, Item 03905). Completely fill the tube by adding equivalent TE/CsCl/EtBr solution (i.e., without DNA) as prepared in the step above, and balance on an analytical scale. Seal the tubes and re-check the balance.

Check each seal by pointing the top of the tube into the sink and applying pressure. Failure to seal tubes appropriately may cause the tubes to collapse during ultracentrifugation.

10. Place the tubes in the ultracentrifuge rotor. Spin at 176,000g for 48 hr at 20°C.
11. Remove tubes from rotor so as to not disturb the gradient. Follow the methods detailed in Sambrook & Russell (2011) to assemble materials used to extract the plasmid DNA. In summary, with an 18g needle make a vent in the tube by puncturing it at the top; leave the needle hanging in the tube to prevent leakage. Using a UV hand lamp (Fisher Scientific, cat. # 95000602) illuminate the tube and carefully pull out the lower plasmid band (the lower band contains the double-stranded plasmid; the upper band contains the single-stranded DNA, Figure 2) with an 18g needle attached to a 1 ml syringe. Place the DNA in a 15 ml Falcon tube.

To avoid DNA shearing during sample collection, remove the needle from the syringe and transfer the plasmid DNA to a clean 50-ml collection tube. Repeat this as many times as necessary.

12. Remove EtBr by adding 2 volumes of isoamyl alcohol, mix well, and centrifuge at 150g for 5 min at room temperature. Remove the upper phase (pink; isoamyl alcohol), and repeat the process until the pink color disappears (3–4 times).
13. Bring the DNA solution to a final volume of 10 ml by adding 10 mM Tris-HCl pH 8.0 (use a 50 ml Falcon tube). Add 1/10 the volume of 3M sodium acetate to the DNA, mix, and add 2.5 volumes of ethanol. Incubate at –20°C for 2hr to overnight.
14. Centrifuge at 10,000g for 45 min at 4°C, wash the pellet with 70% ethanol, and centrifuge at 10,000g for 15 min at 4°C.
15. Discard supernatant. Air-dry the pellet, and resuspend with 500 µl of 10 mM Tris-HCl, pH 8.0.
16. Measure the DNA concentration as previously described in step 7.

Cloning the iPhage vector—To generate the hybrid iPhage vector, we fused the fUSE5 and f88/4 genomes. The fUSE5 vector is engineered to be non-infective by disruption of the gene III reading frame with a 14-bp “stuffer” flanked by *Sfi*I enzyme restriction sites. The replacement of the stuffer by a nucleotide sequence in-frame will result in the expression of

a peptide fused to the rpIII coat protein. The f88-4 vector contains two genes VIII, encoding a wild type (wt) and a recombinant (r) pVIII gene. The rpVIII contains *HindIII* and *PstI* restriction sites that allow directional cloning of foreign peptides displayed in the rpVIII-capsid. The strategy is to generate a bifunctional phage vector containing rpIII and rpVIII, into which a library and the penetratin peptide can be cloned, respectively, and simultaneously expressed in the phage.

17. The oligonucleotides coding the penetratin peptide are commercially obtained (SIGMA) and purified by polyacrylamide gel electrophoresis (PAGE). Resuspend the oligonucleotides with 10 mM Tris-HCl, pH 8.0 at a concentration of 1 µg/µl. Mix equimolar amounts of oligonucleotides (1 µg) in a 0.5 ml microfuge tube in a final volume of 100 µl with 10 mM Tris-HCl, pH 8.0. Incubate the oligonucleotide mix at 100°C for 1 min and slowly cool to room temperature. Store annealed oligonucleotides at -20°C for up to several months.
18. Double-digest the annealed oligonucleotides (1 µg) and f88-4 plasmid (0.5 µg) with *HindIII* and *PstI* restriction endonucleases (5U of each enzyme) in 100 µl overnight at 37°C.
19. Purify the oligonucleotide and plasmid with the QIAquick gel extraction kit (QIAGEN). Analyze the purity of the samples in 2% and 0.8% agarose gels, respectively. To quantify the DNA, use UV-band intensity (100 bp Quanti-Markers) and A₂₆₀.
20. In a 0.5 ml microfuge tube, test various vector:insert molar ratios using 1U of T4 DNA ligase in a final volume of 20 µl, and incubate overnight at 16°C.
The best ratios are between 1:1 and 1:5. During the ligation reaction, we recommend mixing the vector, insert, and water, and incubating at 50°C for 3 minutes, followed by chilling on ice. This procedure improves the ligation efficiency.
21. Add 80 µl of water to each ligation reaction, and take 1 µl for electroporation in DH5α *E. coli*. Add 200 µl of SOC media and incubate for 1 hr at 37°C. Finally, plate different serial dilutions onto LB-tet plates and incubate at 37°C for 24 hr.
22. Pick single bacteria colonies and inoculate in 3 ml of LB-tet (40 µg/ml) media. Incubate overnight at 37°C with gentle agitation (225 rpm).
23. Purify the phage plasmid using the mini-prep plasmid purification kit (QIAGEN). Use 1 µg of plasmid and 1 pmol of each f88-4 sequencing primer (f88/4 fwd 5'- GCTCCTTTCGCTTCTCCCTTCC-3'; f88/4 rev 5'- TCAGGGGAGTAAACAGGAGACAAG-3') for SANGER-based DNA sequencing.
The f88-4 primer set flanks the TAC promoter and the rpVIII gene to confirm the correct peptide-protein fusion in the f88-4/penetratin vector.
24. Individually place 0.5 µg of each plasmid (f88-4-penetratin and fUSE5) in 1.5 ml microfuge tubes containing a double enzymatic reaction mix (5U of *XbaI* and *BamHI*) in a final volume of 100 µl.

25. After 4 hr incubation, load the digested DNA onto a 0.8% agarose gel and run at 100 volts for 45 min. Under an ultraviolet transilluminator, excise the DNA fragment of 3,925 bp from fUSE5 (this contains the rpIII for library cloning) and the 5,402 bp fragment of f88-4/penetratin vector (this contains the rpVIII-penetratin).
26. Place the agarose-DNA fragments in 1.5 ml microfuge tubes and purify each sample using the QIAquick gel-extraction kit (QIAGEN). Prepare a dilution of the DNA and measure the A_{260} .
27. Ligate the DNA fragments using 1:1 molar ratios containing 1U of T4 DNA ligase in a final volume of 20 μ l. Incubate the reaction overnight at 16°C.
28. The ligation products are purified with commercially available desalting and enzyme removal columns (Mini-prep columns, QIAGEN). Elute the DNA with 30 μ l of water and perform the transformation procedure as previously outlined in step 1.

After nucleotide sequencing of individual clones, we recommend making a 30% glycerol-LB bacteria stock and storing it at -80°C . Use proper aseptic technique when handling bacteria glycerol stocks.

Random peptide iPhage library preparation—The fusion of the f88/4 and fUSE5 genomes results in the chimeric vector iPhage (Figure 1). The gene rpIII contains a stuffer DNA that disrupts the open reading frame of the pIII protein. The removal of the stuffer is achieved by the restriction enzyme *Sfi*I, a step that leaves the overhanging sites incompatible for self-ligation and permits directional cloning of a *Bgl*II-digested library insert.

Preparing MC1061 E. coli electrocompetent cells—

29. Inoculate a culture of MC1061 *E. coli* for overnight growth in 10 ml of LB containing 50 $\mu\text{g/ml}$ of streptomycin.
30. Put 2 ml of the seed culture into each of four 2 L flasks containing 500 ml of SOB media with streptomycin (50 $\mu\text{g/ml}$).
31. Monitor the bacterial growth by optical density until absorbance reaches 0.8 at 600 nm (around 3–4 h culture).
32. Centrifuge the bacteria at 6,000g for 10 min at 4°C. Decant and discard supernatant.
33. Wash the bacterial pellet two times with 150 ml ice-cold 10% glycerol.
Keep all solutions cold and maintain the bacteria on ice at all times.
34. Prepare aliquots of 200 μ l or 1 ml in microfuge tubes and snap-freeze in liquid nitrogen.
35. To preserve MC1061 *E. coli* electrocompetency, store aliquots at -80°C for no longer than one week.

36. Test cell competency by electroporating 20 μ l of MC1061 with 10 μ g of high-copy plasmid DNA (e.g. pUC19).

Suitable electrocompetent MC1061 cells should make well above 1×10^9 colonies/ μ g plasmid DNA. The total volume of electrocompetent MC1061 cells needed to generate the iPhage library is 20 ml. To obtain large amounts of MC1061 cells, one may increase proportionally the bacteria culture volume or repeat the steps above as necessary.

Preparing the iPhage vector—The iPhage plasmid is electroporated into DH5 α *E. coli* for maxi-prep plasmid purification and CsCl gradient as previously described (plasmid purification by CsCl gradient steps 4 to 16).

37. Digest 100 μ g of the iPhage vector with 200U of *Sfi*I restriction enzyme in 500 μ l (final volume) for 4 hr at 50°C.
38. Purify the digested iPhage vector with the gel-extraction kit (QIAGEN), which allows the complete removal of the 14 bp DNA stuffer.
39. Check the digestion of the iPhage vector by loading 1 μ l of purified sample (above) onto a 0.8% agarose gel.

Linearized iPhage plasmid may be stored at -20°C for several weeks.

Preparing the insert—The insert library template is commercially purchased as a single-strand degenerate oligonucleotide (PAGE purification grade). The sequence template is X_4YX_4 (X, any residue; Y, tyrosine) configuration: 5'-CACTCGGCCGACGGGGCTNNKNNKNNKNNKTATNNKNNKNNKNNKGGGGCCGCTGGGGCCGAA-3'.

N indicates all four nucleotides; K indicates an equimolar mixture of G and T to prevent the introduction of stop codons into the sequence. Perform 16 PCR reactions to generate enough double strand insert for the ligation reactions.

40. Resuspend the oligonucleotide template and the random library primer (Library sense primer 5'-CACTCGGCCGACG-3', Library antisense primer 5'-TTCGCCCCAGCGGC-3') set with 10 mM Tris-HCl (pH 8.0) for a stock concentration of 1 μ g/ μ l each. Convert the synthetic oligonucleotide template X_4YX_4 , flanked by *Bgl*II restriction sites, to double-stranded DNA by PCR amplification as shown:

Component	Amount per reaction	Final
X_4YX_4 Template	0.1 μ l	100 ng
Library Forward primer	3.0 μ l	3 μ g
Library Reverse primer	3.0 μ l	3 μ g
DMSO	1.0 μ l	2%
10 mM dNTPs	2.0 μ l	0.4 mM

Component	Amount per reaction	Final
25 mM MgCl ₂	2.4 µl	1.2 mM
5× GoTaq buffer	10.0 µl	1×
GoTaq polymerase (5U/µl)	1.0 µl	5U
Milli-Q water	Up to 50 µl	

41. Use the following PCR conditions:

Step	Temperature (°C)	Time	Cycles
Initial Denaturation	94	2 min	1
Denaturation	94	30 sec	
Annealing	60	30 sec	35
Extension	72	30 sec	
Final extension	72	5 min	1

For effective PCR, addition of DMSO (2% final) is recommended, to weaken hydrogen bonding and prevent formation of hairpin structures

42. Purify the PCR products using the nucleotide removal kit (QIAGEN). Measure the DNA concentration at A₂₆₀.
43. Digest 1 µg of library PCR-insert using 100U of *Bgl*I restriction enzyme in a final volume of 50 µl. Incubate overnight at 37°C.
44. Remove enzymes and buffers from the digested PCR-inserts with the nucleotide removal kit (QIAGEN), and quantify the *Bgl*I-digested PCR product as previously described (step 3).

Library ligation - small scale—

45. Determine the optimal vector:insert molar ratio. Perform test ligations with 50 ng of linearized iPhage vector and different *Bgl*I digested PCR-insert ratios (1:1, 1:3, 1:5, and 1:10) as shown below. Incubate the reaction at 16°C for 12 hr. Include a control sample without insert to verify that the vector was completely digested. *The insert-to-vector molar ratio can have a significant effect on the outcome of a ligation and subsequent transformation step.* The formula to calculate the insert amount is:

$$\text{Insert mass(ng)} = \text{Molar Ratio} \times \left(\frac{\text{Insert Length in bp}}{\text{Vector Length in bp}} \right) \times \text{Vector mass(ng)} \quad \text{Eqn. 2}$$

Example for 1:3 molar ratio:

$$\text{Insert mass(ng)} = 3 \times \left(\frac{63\text{bp}}{9234\text{bp}} \right) \times 50 \Rightarrow 1.02\text{ng} \quad \text{Eqn. 3}$$

Component	Amount per reaction	Final
Vector	Depends on concentration	50 ng
Insert	Depends on concentration	e.g. 1.02 ng
5× Ligation buffer	4.0 µl	1×
T4 DNA ligase (5 U/µl)	1.0 µl	5 U
Milli-Q water	Up to 20.0 µl	

To improve the ligation efficiency, mix the vector, insert, and water in a microfuge tube. Next, incubate the mix at 50°C for 3 min and immediately chill the reaction on ice. Proceed with the ligation reaction by adding the ligation buffer and T4 DNA ligase as depicted above.

46. Purify the ligated products on mini-prep columns (QIAGEN) and elute with 30 µl of water.
47. Thaw an aliquot of electrocompetent MC1061 *E. coli* on ice. Place 20 µl of bacteria in a microfuge tube and add 1 µl of the purified ligation product. All these steps must be performed on ice.
48. Transfer the bacteria-DNA mix into an electroporation cuvette (0.1 cm gap; BioRad).
49. Electroporate under the following conditions: 2.0 kV, 250 ohms, 25 µF (Gene pulser II; BioRad).
50. Add 200 µl of SOC media to recover bacteria, and transfer the bacteria into sterile microfuge tubes. Incubate at 37°C for 1 hr under agitation.
51. Plate 1, 10, and 50 µl of transformed bacteria on LB-tet agar plates. Incubate overnight at 37°C.
52. Count the number of colonies and determine the optimal ligation reaction based on the vector-insert molar ratio, transformation efficiency, and background from the negative control ligation.

Library ligation - large scale—

53. Set up the large-scale library ligation reaction as depicted below:

Component	Amount per reaction	Final
Linearized iPhage vector	Depends on concentration	10 µg
Library insert	Optimized quantity from test ligation	
5× Ligation buffer	400 µl	1×
T4 DNA ligase (5U/µl)	100 µl	500U
Milli-Q water	Up to 2,000 µl	

To improve the ligation efficiency, mix the vector, insert, and water in a microfuge tube. Next, incubate the mix at 50°C for 3 min and immediately chill

the reaction on ice. Proceed with the ligation reaction by adding the ligation buffer and T4 DNA ligase as depicted above.

54. Dispense the ligation reaction in 100 μ l aliquots and incubate at 22°C. After 2 hr, transfer the reactions for an overnight incubation at 16°C.
55. Mix the ligation products with 5 volumes of binding buffer (PB buffer) and load the samples into 20 mini-prep QIAprep columns. Let the column stand for 3 min at room temperature.
56. Centrifuge the columns at 10,000g for 30 sec at 4°C. Wash once with 500 μ l of PE buffer, and elute the DNA with 50 μ l of water.

To increase X₄YX₄-iPhage plasmid yield, warm the water to 50°C prior to adding it to the columns.

57. Measure the DNA concentration and perform one thousand electroporations with MC1061 *E. coli* as previously described. Electroporate 10 ng of DNA in 20 μ l of MC1061 per cuvette. Alternatively, a high-throughput electroporation system can be used (ECM 630 + HT-100 BTX Harvard Apparatus). If this method is desired, mix 1 ml of DNA with 22 ml of MC1061 *E. coli*, and let stand on ice for 15 min. Transfer 50 μ l of the mixture into each well of a 96-well high throughput electroporation plate (Multi-well electroporation plate cat. no. 450450, Harvard Apparatus). Electroporate five 96-well plates under the following conditions: 2.4 kV, 750 ohms, 25 μ F.

We obtain approximately 1 ml of purified ligated product. Avoid introduction of air bubbles into the cell/DNA mixture to prevent arcing during electroporation.

58. Combine the electroporations into 400 ml of pre-warmed SOC media and incubate the bacteria under agitation (225 rpm) at 37°C for 1 hr.
59. Add 3.6 L of LB-tet/strep, and divide the culture into 8 baffled Fernbach flasks of 2 L capacity. Incubate the culture overnight at 37°C under agitation (250 rpm).
60. On the next day, transfer the bacterial culture to 400 ml bottles and centrifuge at 6,000g for 15 min at 4°C.

Recover the bacteria pellet, and wash twice with ice-cold 10% glycerol. After the second centrifugation, resuspend pellet in 2 ml of 50% glycerol and aliquot cells into ten chilled 0.5 ml centrifuge tubes (0.2 ml/tube). Snap-freeze pellet in liquid nitrogen and store samples at –80°C. The bacterial pellet is used for library amplification.

61. Transfer the supernatant into clean bottles. Add 45 ml of PEG/NaCl buffer per 300 ml of supernate. Stir supernatant overnight at 4°C.
62. Transfer the precipitation solution equally into ten clean 500 ml centrifuge bottles and centrifuge at 10,000g for 30 min at 4°C. Recover the white phage pellet. Discard the supernatant and centrifuge samples again at 10,000g for 10 min.

Carefully remove the supernatant.

63. Resuspend each pellet in 2 ml of sterile PBS and combine into a 50 ml conical tube. Centrifuge tube at 10,000g at 4°C for 10 min, discard pellet, and transfer iPhage solution into a new 50 ml conical tube.
64. Perform a second phage precipitation by adding 0.15 volumes of PEG/NaCl solution into the iPhage suspension for 1 hr on ice. Centrifuge at 10,000g for 30 min at 4°C. Discard supernatant and resuspend pellet in 0.5 up to 1 ml of PBS depending on the size of the pellet. Store the iPhage library at 4°C.

iPhage particles are relatively stable; the preparations can be stored at 4°C for long periods of time (several months). However, one must know that the titration of an iPhage library should be performed no longer than a week prior to screening.

65. Titrate iPhage by preparing serial dilutions of 10^{-6} , 10^{-7} , and 10^{-8} of the phage library in PBS (10 μ l/dilution). Prepare dilutions in triplicate. For each 10 μ l of dilution add 100 μ l of log phase K91/kan *E. coli*. Allow iPhage infection for 30 min at room temperature. Plate 100 μ l of each dilution in triplicates on LB plates containing tetracycline (40 μ g/ml) and kanamycin (50 μ g/ml), and incubate at 37°C overnight. The iPhage titers are expressed as bacterial TUs/ μ l. Calculate iPhage titer using the following formula:

$$\text{Phage titer (TU}/\mu\text{l)} = \left(\frac{\text{no. of colonies counted}}{10} \right) \times \text{dilution factor}$$

For example, if 100 colonies are counted in the 10^{-7} dilution plate:

$$\text{Phage titer} = \left(\frac{100}{10} \right) \times 10^7 \Rightarrow 1 \times 10^8 \text{ TU}/\mu\text{l}$$

K91/kan E. coli viability plays an important role in iPhage titration. Always infect a log-phase growing bacteria with an optical density ranging between 1.6 and 2.0 at a wavelength of 600 nm (OD_{600}).

BASIC PROTOCOL II

SCREENING, SELECTION, AND RECEPTOR VALIDATION OF CANDIDATE IPHAGE CLONES

iPhage technology can be applied to uncover in an unbiased manner intracellular pathways, intracellular protein-protein complexes, and organelle receptors in their native conformation. By separation of the nuclear, cytosolic, and mitochondrial fractions after phage library panning, bioactive phage particles and therefore their peptide sequences can be identified and characterized according to their sub-cellular niche (Figure 3). Ultimately, bioactive intracellular peptide ligands identified by iPhage can be tailored to other targeting entities for tissue selectivity.

Although the Kaposi Sarcoma (KS) 1767 cell line was used in the original manuscript (Rangel et al., 2012), the iPhage screening and selection can be performed in virtually any cell line. In fact, the iPhage applicability is not limited to species (mouse or human), transformation status (non-malignant or malignant cells), nor tumor type (carcinoma, leukemia, lymphoma, melanoma, or sarcoma). The approach used to identify binding iPhage particles consists essentially of 3 basic steps: (i) introduction of iPhage particles to an immobilized target (i.e. KS1767 cell line), (ii) removal of unbound iPhage, and (iii) elution of bound iPhage particles. However, one should note that because the entire iPhage library enters the target cell, the identification of specifically bound iPhage will require accurate isolation of organelles to determine enrichment of specific peptide sequences in particular sub-cellular fractions.

To isolate target-specific iPhage binders, one cycle of in vitro selection should in theory be sufficient, yet, in practice, several rounds of selection are actually necessary - typically two to four (Figure 2). The Basic Protocol II described below encompasses the method of iPhage selection, subcellular fractionation process and recovery of iPhage bound particles, identification of iPhage clones, and crafting of affinity chromatography for receptor isolation and characterization, as applied to the selection of iPhage clones recognizing mitochondrial targets. Similar approaches for iPhage selections targeted to different organelles can be easily carried out, requiring purification of those organelles using standard techniques. In order to validate candidate receptors, phage binding and immunocapture assays are often used and therefore also explained in this section.

Materials—Kaposi Sarcoma 1767 cell line

75 and 175-cm² tissue culture flasks (BD Biosciences)

Minimal Essential Medium (MEM; Cellgro)

Fetal Bovine Serum (FBS; Life Technologies)

MEM-vitamin solution (100X, Life Technologies)

MEM Non-essential amino acids solution (100X, Life Technologies)

Penicillin-Streptomycin solution (100X, Life Technologies)

L-Glutamine (100X, Life Technologies)

Syringe filter (0.22 and 0.45µm; Millipore)

0.05 % Trypsin-EDTA (1X, Life Technologies)

Hypotonic buffer (APPENDIX)

Dounce homogenizer (Fisher Scientific)

2.5× stabilization buffer (APPENDIX)

Parafilm (Fisher Scientific)

2 ml microfuge tubes

1X Mitochondria stabilization (MS) buffer (APPENDIX)

1M Isopropyl β -D-1-thiogalactopyranoside (IPTG) stock (SIGMA)

96-well U-bottom plates (BD Biosciences)

96-well PCR plates (Eppendorf)

pIIIseq Forward: 5'-AGCAAGCTGATAAACCGATACAATT-3' (DESALTED)

pIIIseq Reverse: 5'-CCCTCATAGTTAGCGTAACGATCT-3' (DESALTED)

96- well microplates flat bottom (BD Biosciences)

Phase contrast microscope

Cell proliferation kit (MTT or WST-1 assays, Roche)

2 mM EDTA-PBS

Protein extraction buffer (APPENDIX).

Synthetic peptides used for elution (5 mM in column buffer)

CarboxyLink Immobilization Kit (Thermo Scientific)

Column buffer (APPENDIX)

Elution buffer (column buffer with 5mM peptide)

Glycine buffer (APPENDIX)

0.05% Sodium azide-PBS (APPENDIX)

50 mM Octylglucoside-PBS

Dialysis cassettes (Thermo Scientific)

Spin column concentrators (Thermo Scientific)

BCA protein assay kit (Thermo Scientific)

0.1, 1, and 2% Bovine serum albumin (BSA)

Terrific broth (TB, APPENDIX)

2X Laemmli buffer (Bio-Rad)

4–20% SDS-PAGE gels

Coomassie blue stain (SimplyBlue SafeStain, Life Technologies)

Stainless-steel blade (Fisher Scientific)

Protein A-coated 96-well plates (Thermo Scientific)

First round of selection—

1. Grow Kaposi Sarcoma (KS) 1767 cell line to 50% confluence in 75-cm² culture flasks. The cells are cultured in MEM containing 10% FBS, MEM-vitamins, non-essential amino acids, penicillin G (100U), streptomycin SO₄ (100 µg/ml) and 2.7 mM L-glutamine at 37°C in a 5% CO₂-humidified incubator.
2. Prepare 5×10¹¹ TU of the iPhage library in 8 ml of MEM containing 10% FBS. Filter the iPhage library solution (8 ml) through a 0.22 µm filter. Remove the media from the overnight culture and add the iPhage library. Incubate overnight at 37°C in a 5% CO₂-humidified incubator.
3. On the following day, remove and discard iPhage media from the culture flask with a sterile pipette. Wash cells extensively with 20 ml of pre-warmed PBS to remove any remaining media. Detach the cells with 3 ml of 0.05 % trypsin-EDTA (Invitrogen), and incubate for 10 min at room temperature.
4. Neutralize the trypsin by adding 10 ml of MEM containing 10% FBS. Transfer the cell suspension into a 15 ml tube.
5. Centrifuge cells at 150g for 5 min at room temperature. Decant and discard supernatant. Wash the cell pellet three times with ice-cold PBS to remove traces of trypsin/EDTA and growth media. Resuspend pellet in 1 ml of PBS.

Subcellular fractionation and recovery of iPhage bound particles from mitochondrial selections—

6. Mix the pellet suspension (1 ml) with 11 ml of ice-cold hypotonic buffer. Transfer cell suspension into a Dounce homogenizer and incubate for 10 min on ice.
7. Perform 15 up-and-down strokes with a loose-fitting pestle and 25 up-and-down strokes with a tight-fitting pestle on the KS1767 cells to disrupt the membranes, and add 8 ml of 2.5× MS buffer. Seal the tube with parafilm and mix thoroughly by inversion.
8. Aliquot the 20 ml cell lysate suspension into ten 2 ml ice-cold microfuge tubes, and centrifuge at 1,300g for 5 min at 4°C. Recover and transfer supernatant into clean microfuge tubes. Centrifuge samples twice more. The pellet contains the nuclei, intact cells, and large membrane fragments.
9. Transfer the supernatant into a clean 2 ml microfuge tube and repeat the nuclear spin-down twice at 1,300g for 5 min (4°C).

10. Transfer supernatant into clean 2 ml microfuge tubes and pellet the mitochondria at 17,000g for 15 min at 4°C. Decant and discard supernatant.
11. Wash the mitochondria by resuspension of the pellet in 500 µl MS buffer. Centrifuge samples once more at 17,000g for 15 min at 4°C. Discard the supernatant and keep the mitochondrial pellet on ice.
The mitochondrial pellet can be stored at –80°C for further assays.
12. If the iPhage recovery is performed on the same day, we recommend starting a K91/kan *E. coli* culture before the subcellular fractionation. Inoculate K91/kan bacteria in TB medium containing supplements, and 100 µg/ml kanamycin, and incubate at 37°C under agitation (225 rpm). After 2–3 hr, use a 1:10 dilution to measure A₆₀₀ (it must reach 0.180 to 0.200 absorbance for infection of the subcellular fraction.)
13. Add 200 µl of the K91/kan *E. coli* onto each mitochondrial pellet aliquot (10 aliquots total – step above). With a micropipette gently resuspend and mix the pellet. Incubate sample for 1 hr at room temperature.
14. Transfer the 200 µl of bacteria (above) to 10 ml of pre-warmed LB medium supplemented with 40 µg/ml tetracycline and 100 µg/ml kanamycin, and incubate for 30 min at room temperature.
15. Dilute sample to 1:10, 1:100, and 1:1,000, plate 100 µl of each dilution in triplicate onto LB-tet/kan plates, and incubate plates overnight at 37°C.
16. After plating the bacteria, transfer the rest of the culture (~10 ml) into 300 ml of pre-warmed LB tet/kan containing 1 mM IPTG and incubate overnight at 37°C.

iPhage precipitation—

17. On the next day, centrifuge the bacterial culture at 6,000g for 20 min at 4°C, and transfer the supernatant into new bottles containing 45 ml of PEG/NaCl solution; incubate 2 hr on ice.
18. Centrifuge the phage solution at 10,000g for 30 min at 4°C. Discard the supernatant and centrifuge again for 10 min. Carefully remove the remaining supernatant.
19. Resuspend the iPhage pellet with 1 ml of sterile PBS and incubate at 37°C for 10 min under agitation (250 rpm). Repeat the precipitation with 0.15 volumes of PEG/NaCl. Incubate on ice for 1 hr.
20. Centrifuge at 10,000g for 30 min at 4°C and resuspend the pellet with 100–300 µl of PBS (depending on the mass of the precipitated phage).
21. Remove any insoluble material by centrifuge at 14,000g for 10 min at 4°C. Transfer supernatant to a new sterile 1.5 ml microfuge tube.
22. Filter the iPhage particles through a 0.22 µm filter and titrate the iPhage particles as previously described (first round of selection step 2).

The recovered iPhage particles are stable for at least two weeks without reduction in titer.

PCR-sequencing of iPhage insert—After three rounds of iPhage selection, pick 96 bacterial colonies from each round to determine the amino acid sequence of the recovered iPhage clones. The peptide identity is determined by sequencing the DNA corresponding to the insert in the iPhage genome (pIII).

23. Prepare three 96-well U-bottom plates with 50 μ l 30% glycerol-LB. Pick well-separated bacterial colonies from the agar plates of each round of selection.

Plates may be stored at -80°C for later analysis.

24. Prepare a PCR reaction as described below. For high throughput screening use a 96-well PCR plate. Include a negative control of an iPhage without insert.

Component	Amount per reaction	Final
Bacterial suspension	2.0 μ l	
pIIIseq forward primer	1.0 μ l	8 pmol
pIIIseq reverse primer	1.0 μ l	8 pmol
10 mM dNTP	0.5 μ l	0.25 mM
5 $\times$ GoTaq buffer	4.0 μ l	1 $\times$
DMSO	0.4 μ l	2%
GoTaq polymerase (5U/ μ l)	0.4 μ l	2U
Milli-Q water	Up to 20 μ l	

25. Use the following PCR conditions

Step	Temperature ($^{\circ}\text{C}$)	Time	Cycles
Initial Denaturation	94	3 min	1
Denaturation	94	10 sec	
Annealing	60	30 sec	35
Extension	72	1 min	
Final extension	72	3 min	1

26. Prepare a 2% agarose gel and run 2 μ l of ten PCR reactions randomly selected from each PCR plate.

Positive iPhage colonies will have PCR products of around 300 bp, whereas the negative control will generate a PCR product of around 250 bp.

27. Make a 1:10 dilution with water from each PCR reaction and submit samples for nucleotide sequencing with the pIIIseq reverse primer. The peptide sequences and sequence enrichment are analyzed based on bioinformatics tools and are described elsewhere (Dias-Neto et al., 2009).

Amplifying iPhage clones—

28. iPhage clones of choice can be amplified by inoculation of 3 ml of LB tet-strep containing 1 mM IPTG with 1 μ l of the bacteria glycerol stock (step above) and incubation of the seed culture at 37°C for 8 hr.
29. Transfer the seed culture to 100 ml of LB tet-strep containing 1 mM IPTG and follow the iPhage-precipitation protocol as previously described.
30. Titrate each iPhage clone no longer than a week prior to performing the cell viability assay. Filter the iPhage clone through 0.22 μ m.

Cell viability assay—

31. Seed 2.5×10^4 KS1767 cells in each well of a 96-well flat-bottom tissue culture plate in a final volume of 100 μ l of complete growth media.
32. Incubate the cell culture overnight at 37°C in 5% CO₂.
33. After the incubation period, remove growth media and avoid disturbing the cell monolayer. Carefully add 100 μ l of complete growth media containing 1×10^9 TU of iPhage in triplicate and incubate plate overnight at 37°C in 5% CO₂.

Include insertless-iPhage and parental phage as negative controls as well as the YKWYYRGAA-iPhage as a positive control (Rangel et al., 2012).

34. After 24 hr, take 10 random phase-contrast microscopy images from each triplicate and determine cell viability by addition of 10 μ l of MTT (12 mM) according to the manufacturer's instructions.
35. Mix each sample thoroughly with a micropipette and read absorbance at 570 nm. Plot data and correlate with the cell density/morphology images obtained with phase-contrast microscopy.

Bioactive iPhage can be selected based on reduced cell growth rate and cell viability measured by MTT or WST-1 assays as well as cell shrinkage or pyknosis (chromatin condensation) visible by light microscopy.

Ligand receptor purification by affinity chromatography—Affinity columns are crafted by coupling of the selected synthetic peptide to agarose or magnetic beads. We recommend using a cell lysate of the same cell line used in the iPhage screening. The cell lysate is loaded onto the column and the receptor is eluted with the corresponding competitive peptide at high concentration (e.g. 5 mM). Eluted fractions are monitored by absorbance (optical density at 280 nm), desalted, and concentrated.

Cell lysate preparation—

36. Grow KS1767 cells to 90% confluence in six 175-cm² culture flasks. One day prior to use, add new media and incubate overnight at 37°C in a 5% CO₂.

37. Wash the cells with 25 ml ice-cold PBS and aspirate the PBS. Detach the cells in 10 ml of ice-cold 2mM EDTA in PBS, and incubate on ice for 10 min. Gently, tap the flask to facilitate cell detachment.
38. Transfer the cell suspension into 50 ml tubes and centrifuge at 150g for 3 min at room temperature.
39. Wash the cell pellet once with plain MEM and resuspend the pellet in a 1:1 volume of ice-cold extraction buffer.
40. Incubate the cell lysate at 4°C for 1 hr on a rocking plate.
41. Centrifuge the cell lysate at 20,000g for 15 min at 4°C. Keep the supernate on ice until use. Check that the cell extract does not contain any precipitated material.

Affinity chromatography—The carboxylink immobilization kit (Thermo Scientific) contains crosslinked-agarose beads. One day before the cell lysate preparation (depicted above), conjugate the peptide of choice to the agarose column matrix according to the protocol supplied by the commercial vendor.

42. Conjugate 10 mg of peptide to a 2 ml agarose column matrix (Carboxylink immobilization kit), according to the manufacturer's instructions.
43. Equilibrate the affinity column and column buffer to room temperature. Equilibrate column by passing 10 ml of column buffer through the column.
Throughout the procedure, do not allow the resin to become dry. Replace bottom cap as soon as buffer drains down to the top of the resin bed.
44. Carefully apply 1–2 ml of cell lysate (depending on the size of the cell pellet) into the column. Cap and seal the column at both ends with parafilm, and rock for 1 hr at room temperature.
45. Place the column in a base support stand, remove top and bottom caps from column, and wash the column with 20 ml of column buffer. Monitor the absorbance at 280 nm until it reaches 0.001. To remove any residual protein, wash the column with 10 ml of column buffer.
46. Apply 2 ml of peptide-ligand (5 mM) in column buffer and collect 0.5 ml fractions. Continually add 20 ml of column buffer to the column and monitor elution by absorbance at 280 nm until it reaches 0.001 (approx. 20 samples).
The excess of bioactive peptide will compete with the protein complex associated with the peptide crosslinked to the column and will thereby release the protein receptor.
47. Remove all remaining protein complexes by adding 10 ml of glycine buffer. Recover 10 fractions of 1 ml each.
48. Wash the column with 10 ml of column buffer, and equilibrate the column with 0.05% sodium azide-PBS.

Keep the column at 4°C. Affinity columns are stable for 12 months when they are properly washed and stored. Sodium azide is a common preservative of samples and stock solutions; because it is a hazardous chemical, wear gloves and facemask during the preparation of solutions.

49. Place the eluted samples on ice. Load slide-A-lyzer cassettes 3 kDa (Pierce) with either the peptide-eluted or glycine fractions and dialyze overnight against 50 mM octylglucoside-PBS at 4°C. Follow the manufacturer's instructions for loading of the dialysis device.
50. Collect and concentrate the samples with spin column concentrators 3 kDa (PIERCE). All steps are performed at 4°C. Measure protein concentration with the BCA kit (Pierce). Store protein samples at -80°C.

Phage-binding assay—This assay allows the identification of eluted fractions containing the intracellular/organelle receptor for further biochemical characterization. It consists of i) coating a 96-well plate with equal amounts of eluted protein fractions, ii) incubation with targeted iPhage or parental insertless iPhage, and iii) recovering the phage by K91/kan bacterial infection. With this protocol the exact receptor distribution can be determined by bacterial colony counting. We recommend titering the phage one day before the binding assay to obtain an accurate estimate of phage input in the assay.

51. Immobilize 5 µg of protein diluted in PBS (pH 7.2) from each fraction of interest in triplicate on a 96-well flat-bottom plate. As a negative control, immobilize 50 µl of 1% (wt/vol) BSA in PBS. Incubate the plate overnight at 4°C.
52. The next day, wash the wells once with 200 µl of PBS.
53. Block each well with 200 µl of PBS containing 1% (wt/vol) BSA and incubate at room temperature for 2 hr.

To reduce non-specific phage binding with BSA, filter the blocking solution through a 0.22 µm filter before use.

54. Wash twice with 200 µl of PBS. Add the washing solution slowly.
55. Add 1×10^9 iPhage particles/well of bioactive iPhage clone or insertless iPhage diluted in 50 µl of PBS containing 0.1% (wt/vol) BSA into wells containing the fraction of interest and negative control wells. Incubate plate at room temperature for 2 hr.
56. Wash the 96-well plate 12 times with 200 µl of PBS.
57. Remove the PBS and add 200 µl of K91/kan *E. coli* (grown in TB/kan at log phase, $A_{600}=0.2$) for 1 hr at room temperature.

K91/kan E. coli must be grown on the day of panning. For infection use log-phase growing bacteria with an optical density ranging between 1.6 and 2.0 at a wavelength of 600 nm (OD_{600}).

58. Transfer bacteria from each replicate well into a 2 ml microfuge tube and dilute to 1:5, 1:20, and 1:200.
59. Plate 100 μ l on kan/tet plates in triplicate from each dilution and incubate overnight at 37°C. Count bacterial colonies on the following day. The elution fraction containing the most colonies represents the fraction containing the receptor for the ligand-peptide.
60. Identify the fraction containing the candidate receptor. Prepare 5 – 20 μ g of protein with 4 $\times$ NuPAGE LDS sample buffer containing 2-mercaptoethanol from a negative control fraction (low or no colonies) and from the fraction of interest. Load protein samples onto a precast Novex 4–20% Tris-glycine gel and run at 200 volts for 50 min.

2-mercaptoethanol is harmful if swallowed. It is toxic in contact with skin and causes burns. Work in a fume hood and wear proper protective equipment.

61. Wash the gel twice with 200 ml of water for 5 min and stain with Coomassie blue for 1 hr at room temperature. Photograph and scan the gel to detect the protein bands corresponding to the receptor. Use a new scalpel blade to cut and transfer the acrylamide fragments to a 1.5 ml microfuge tube.
62. Request mass spectrometry analysis of the samples.
In some cases, the purified fractions contain multiple bands difficult to extract. In such cases, it is necessary to perform two-dimensional gel electrophoresis.

Receptor validation—The mass spectrometry analysis provides a list of candidate receptors that need experimental validation. Use a phage binding on recombinant protein or an immunocapture assay to validate the receptor-ligand interactions. It is recommended to use 3 to 5 candidate receptors in the assay. If recombinant proteins are not available, use antibodies to immunocapture these proteins from the cell lysate.

Phage binding assay with recombinant proteins—

63. Immobilize 0.1–0.5 μ g of recombinant protein in 50 μ l of PBS in triplicate on a 96-well flat-bottom plate. If recombinant proteins are tagged (e.g. with GST), use the tag alone as a negative control. Otherwise, immobilize 50 μ l of 1% (wt/vol) BSA in triplicate. Incubate the plate overnight at 4°C.
64. On the next day, wash plate once with 200 μ l of PBS.
65. Block the well with 300 μ l of 1% BSA-PBS, and incubate for 1 hr at room temperature.
66. Wash plate once with 200 μ l PBS.
67. Add 5×10^9 TU of iPhage clone in 50 μ l of 0.1% BSA-PBS. Incubate for 2 hr at room temperature.
68. Wash 10 times with 200 μ l of PBS.

69. Add 200 μ l of K91/kan *E. coli* to each well and incubate for 1 hr at room temperature.
70. Transfer the bacteria to 0.8 ml of LB kan/tet (0.4 μ g/ml) and make serial dilutions (1:10, 1:100, 1:1000). Plate 100 μ l on LB-agar kan/tet plates in triplicate from each dilution and incubate overnight at 37°C. Count bacterial colonies on the following day and plot the data.

Immunocapture assay—

71. Use protein A-coated 96-well plates and add 10 μ g/ml of desired antibody (polyclonal or monoclonal) in triplicate. Alternatively, dilute antibody 1:100 in 50 μ l of PBS. As a negative control, add 10 μ g/ml or equal dilution of immunoglobulin isotype control in triplicate. Leave at least 3 empty wells (i.e. without antibodies) to be used as 'blank' (e.g. BSA-blocked protein A coated wells). Incubate plate overnight at 4°C.
72. Next day, add 300 μ l of blocking solution (2% BSA-PBS) and incubate for 2 hr at room temperature.
73. Add the cell extract containing 30–60 μ g of protein in 50 μ l of PBS into each well and incubate overnight at 4°C.
74. Remove the plate from 4°C and leave it at room temperature for 1 hr.
75. Wash 3 times with 200 μ l of PBS.
76. Add the desired iPhage clone (1×10^9) diluted in 50 μ l of 0.1% BSA-PBS. Incubate for 2 hr at room temperature.
77. Wash 11 times with 200 μ l of PBS.
78. Add 200 μ l of K91/kan bacteria and incubate for 1 hr at room temperature.
79. Transfer the bacteria to 0.8 ml of LB kan/tet (0.4 μ g/ml) and make serial dilutions (1:10, 1:100, 1:1000). Plate 100 μ l on LB-agar kan/tet plates in triplicate from each dilution and incubate overnight at 37°C. Count bacterial colonies on the following day and plot the data.

These assays are commonly used to validate candidate receptors for peptides isolated from the combinatorial phage display screenings. There are several factors that influence the results of the assays: e.g., the amount of immobilized protein, the affinity of the peptide, conformation of the protein, the existence of protein complexes, temperature, and ionic strength. These aspects must be taken into consideration during the experimental procedure.

REAGENTS AND SOLUTIONS

Super optimal Broth (SOB) Media—For 1 liter, dissolve 20g bacto tryptone, 5g bacto yeast extract, 2 ml of 5M NaCl, 2.5 ml 1M KCl, 10 ml of 1M MgCl₂, and 10 ml of 1M MgSO₄ in 900 ml of distilled water and adjust to 1 L with distilled water. Sterilize by autoclaving. Store at room temperature.

Super Optimal Broth with Catabolite repression (SOC)—Follow the steps above and add 20 ml of 1M glucose. Sterilize by autoclaving. Store at room temperature.

Terrific broth (TB)—Dissolve 12 g tryptone, 24 g yeast extract, and 4 ml glycerol in 900 ml of distilled water. Sterilize by autoclaving and cool to room temperature. Adjust volume to 1000 ml with 100 ml of a filter-sterilized solution of 0.17M KH_2PO_4 and 0.72M K_2HPO_4 . Store at room temperature.

Luria-Bertani (LB) medium and agar plates—For 1 liter, dissolve 10g tryptone, 5g yeast extract, 15g agar, and 10g NaCl in 950 ml deionized water. Adjust the pH of the solution to 7.0 with NaOH and bring the volume up to 1 liter. Autoclave in liquid cycle for 20 min at 15 psi. Allow the solution to cool to 55°C, add antibiotic (i.e., kanamycin, streptomycin, tetracycline) and pour into 10 cm plates. Let harden, invert, and store at 4°C in the dark.

Kanamycin stock—Dissolve kanamycin (kanamycin monosulfate; Sigma-Aldrich, Cat No. K4000) at 50 mg/ml in distilled water and filter sterilize. Divide into 1 ml aliquots and store in the dark at –20°C. Working concentration is usually 50 µg/ml.

Streptomycin stock—Dissolve streptomycin sulfate (Cat. No. S6501, Sigma-Aldrich) in water for a 50 mg/ml stock, and filter sterilize. Store at –20°C. Working concentration is usually 50 µg/ml.

Tetracycline stock—Dissolve 20 mg/ml of tetracycline (Sigma-Aldrich, T8032) in ethanol. Store at –20°C in the dark. Working concentration is usually 40 µg/ml.

Polyethylene glycol (PEG)-Sodium chloride (NaCl) (16.7% 13.3M stock)—Dissolve 100g of polyethylene glycol-8000, and 116.9g of NaCl in 475 ml water. Adjust volume to 600 ml. Warm the solution up to 65°C to dissolve solids. Alternatively, the solution can be autoclaved, then agitated while hot until the liquid cools to room temperature. Store at 4°C.

Hypotonic buffer—10 mM NaCl

1.5 mM MgCl_2

10 mM Tris-HCl (pH 7.5), store at 4°C.

2.5X Mitochondrial stabilization (MS) buffer—525 mM mannitol

175 mM sucrose

2.5 mM EDTA (pH 7.5)

12.5 mM Tris-HCl (pH 7.5), store at 4°C.

Protein extraction buffer—1mM CaCl_2

1mM MgCl₂

50 mM octylglucoside

0.2 mM phenylmethylsulfonyl fluoride (PMSF)

Protease inhibitor cocktail (1 tablet per 50 ml, Roche)

1% Triton X-100 in PBS. The protein extraction buffer can be stored at –20°C, without PMSF and protease inhibitors.

Column buffer—0.01mM CaCl₂

0.01mM MgCl₂

50 mM octylglucoside

0.2 mM PMSF

1 tablet of protease inhibitor cocktail (Roche) per 50 ml of PBS. Prepare the buffer only as needed.

Glycine buffer—100 mM Glycine

100 mM NaCl (pH 3.0)

Store at room temperature

COMMENTARY

Background information

Targeted phage constructs that penetrate eukaryotic cells through a receptor-independent mechanism provide a novel discovery platform for the selection, evaluation, and validation of intracellular molecular interactions in live cells. In this protocol, we introduce the conceptual design, generation, and initial development of iPhage as a new biotechnology resource for combinatorial targeting and discovery of intracellular- and/or organelle-receptors in mammalian cells (Rangel et al., 2012).

Phage display is a system for the high throughput analysis of protein interactions and thus is a powerful proteomics technology. Importantly, this technology allows the unbiased identification of receptors in their native conformation, which may not be preserved outside the context of intact cells during related techniques such as affinity chromatography. Over the past two decades, phage selection *in vitro* and *in vivo* has consistently contributed to our understanding of cell-surface biology by revealing novel functions for known proteins, novel multi-protein complexes, and targetable expression patterns in pathologic settings, which have been discovered by our own group (Pasqualini and Ruoslahti, 1996; Arap et al., 1998; Pasqualini et al., 2000; Arap et al., 2002a; Mintz et al., 2003; Kolonin et al., 2004;

Kolonin et al., 2006a; Mintz et al. 2009; Staquicini et al., 2011) and others (Laakkonen et al., 2002; Higgins et al., 2004; Ballard et al., 2006; Zhang et al., 2006; Hardy et al., 2008).

In contrast to yeast (two) hybrid systems, where protein interactions are assessed under physiological (i.e., *in vivo*) yet artificial (gene fusion) conditions, phage display can be readily modified to manipulate selection conditions and stringencies, allowing for the rapid screening of large numbers of proteins against potential binding partners. In fact, iPhage may be combined with receptor-targeting peptides, which provide tissue selectivity, and with intracellular bioactive peptides discovered by iPhage. After specific delivery, such constructs have the potential to modulate cell function in a tissue- and/or organ-specific fashion. The new resource introduced here can target intracellular 'ZIP' codes, interrogate signal transduction pathways, and participate in developing an organelle-targeted cell biology and pharmacology in mammalian cells.

Critical parameters and Troubleshooting

Generation of the internalizing phage peptide library—The number and diversity of individual clones present in a given peptide phage-display library is approximately 10^9 . This means that the diversity of the peptide sequences present in a library is limited. If the random insert is seven residues or longer, only a portion of all possible permutations of such peptides is actually displayed. Therefore, the preparation of the library is a critical factor and has to be optimized such that the number of recombinants is as high as possible. To that end, we recommend purifying the f88-4 and fUSE5 phage plasmids by a cesium chloride gradient. Although this method is more labor-intensive, it yields high-quality plasmid DNA free of most contaminants. Drawbacks of CsCl-EtBr centrifugation are the long spin times and the use of EtBr, which must be disposed of by charcoal filtration [i.e., funnel kit, the green bag kit (VWR)].

Another critical step is the preparation of the MC1061 *E. coli* competent cells. Proper aseptic techniques must be used when handling bacteria. All solutions must be sterile-filtered (0.22 μ m). The 10% glycerol solution should be kept on ice during the washes, and bacteria are frozen immediately in liquid nitrogen. To manipulate liquid nitrogen use hand protection and goggles, and use only containers designed for extreme cold.

Lastly, we advise that DNA fragments (i.e., vector, insert) are added to the tubes together with water and then warmed to 50°C for 3 min in order to melt any cohesive termini that have reannealed during the DNA fragment purification. Additionally, purification of the ligated products and recovery in water permit high electroporation efficiency in the MC1061 bacteria strain.

Screening, selection, and receptor validation of candidate iPhage clones—One day before the screening experiment, it is strongly recommended to titer the phage library to determine the number of phage particles in the cell culture. Use only sterile single-use or autoclaved glassware and avoid spills. After handling phage purifications, kill bacteria and phage by adding 10 % bleach/water and incubating for 30 min before disposal in the sink.

Fresh solutions for the subcellular fractionation should always be considered. The fractionation buffers contain a carbon source that favors bacteria growth; therefore, filter-sterilization is recommended. However, these solutions may be stored at 4°C for no longer than one week.

Importantly, every cell line used for iPhage screening must be tested for mycoplasma in order to avoid unreliable experimental data. We recommend subculture of the cells for 3 to 5 passages before addition of the iPhage particles. During the subcellular fractionation all the steps are performed at 4°C.

For phage binding assays, several factors are key to the process of receptor validation. First, the cell lysates must be prepared with non-denaturing detergents and protease inhibitors, to maintain native conformation and/or protein complexes, and to prevent protein degradation, respectively. During the affinity chromatography step, we have purified the candidate receptor in association with other molecular components that had previously been reported to form protein complexes (Rangel et al., in preparation). Mass spectrometry data and bioinformatics analysis (i.e., DAVID, String, ingenuity pathway analysis), should indicate whether the purified proteins are involved in molecular complexes.

Receptor validation assays are challenging due to temperature, ionic strength, and conformation, which might influence the molecular interaction between the peptide and receptor. Thus, we recommend the use of PBS and Tris-buffered saline for the phage binding assays. The blocking solution (BSA) should be filtered (0.22µm). Although simple, this step minimizes nonspecific control phage binding and bacterial colony background.

Finally, in the immunocapture assay a critical factor is the determination of the expression levels of the candidate receptor. Lysates obtained from the cell line screened should be analyzed by Western blot to define the protein extract source. In addition, different concentrations of immobilized antibodies and cell extracts should be incubated to determine optimal conditions for the immunocapture assay. It is important to ascertain the amount of immobilized protein on the plate. The best protein concentration to be used can be easily estimated by Bradford or BCA assay.

iPhage Peptide Library Analytics—The iPhage peptide library is subjected to the workflow summarized in Figure 4. All sequences are first evaluated against literature-based resources, namely the dedicated peptide resource PepBank [<http://pepbank.mgh.harvard.edu/>] (Duchrow et al., 2009) and the general bioactivity platform CARLSBAD [<http://carlsbad.health.unm.edu/>] (Mathias et al., 2013). First, we reduce the sequences to motifs by clustering the 20 proteinogenic amino acids into groups of 1 or more similar residues, using 5 bioinformatic and cheminformatic criteria. These motifs are subject to systematic enumeration of all possible sequences, from single-residue up to 8-mer sequences, including gaps and reverse sequences.

All matched motif-sequence associations are then scored using a Bayesian Smoothing technique by taking into account both matched sequence counts and the number of unique sequences matched by that specific motif. Of the exhaustively generated motifs, the top 100

scored, together with the matched sequences and their corresponding counts are analyzed for occurrence in specific tissues / locations. Preference is given to those that occur selectively in a single tissue sample. Selected peptide sequences are subject to synthesis and biological testing.

Anticipated results—If the iPhage constructs are properly assembled, generation of iPhage particles is often abundant and non-toxic to the host bacteria K91/kan *E. coli*. iPhage library titers should range from 1×10^{10} to 5×10^{10} TU/ml.

Three rounds of synchronous selection are usually performed to isolate intracellular- or organelle-enriched iPhage clones. After round 3, a significant increase in iPhage particles should be observed in each of the subcellular fractions. Appropriate bioinformatic analyses must be applied to identify enriched sequences. The selected peptide candidate is synthesized by solid-phase method and used in functional assays as well as for receptor identification.

After the peptide candidate is characterized *in vitro*, the peptide is coupled to agarose beads and utilized to affinity-purify its binding partner or intracellular receptor. Fractions obtained from the affinity chromatography are individually tested against the iPhage candidate. Positive fractions are further analyzed by SDS-PAGE or alternatively by two-dimensional gel electrophoresis, to identify a distinct set of proteins present in the peptide eluate. Unique bands should be methodically and carefully isolated, and analyzed by tandem mass spectrometry (MS/MS) to reveal a list of reasonable receptor candidates. The use of bioinformatic tools (e.g., DAVID, String, or IPA) to mine the MS/MS data is encouraged and should be performed at an early stage of the investigation.

In summary, our method allows an unbiased combinatorial selection, isolation, identification, and validation of intracellular receptors directing iPhage particles or ligand peptides to distinct and specific cell compartments.

Perspective for Nanotherapeutics—A critical and compelling question is whether iPhage identified peptides could be implemented on nanoparticles to direct them to intracellular targets. It is now widely recognized/appreciated that targeted delivery of drugs and/or imaging agents packaged within nanoparticles (NPs) could be transformative for cancer therapeutics and diagnostics as the NP can protect delicate cargos and confer new ‘effective solubilities’ allowing delivery of currently undeliverable cargos based on their physicochemical parameters such as size, charge, and hydrophobicity (Davis et al., 2008). Additionally targeted delivery of cargo to a target cell, while avoiding non-specific binding to normal cells, would avoid harmful side effects of conventional chemotherapy.

An ideal targeted nanoparticle drug carrier, or “nanocarrier” should have the: 1) capacity for carrying high levels of multiple diverse molecular cargos (small molecules, drugs with varying physicochemical properties, siRNAs, peptides, imaging agents); 2) ability to circulate in the blood *in vivo* for extended periods without elimination by the immune or excretory systems; 3) specificity for binding only to target disease cells or tissues, while avoiding normal, healthy cells; and 4) low immunogenicity and toxicity. To date targeted nanocarriers

have been developed using multiple types of materials including biodegradable polymers, liposomes, inorganic nanoparticles (metals, semiconductors, and oxides) and carbon-based materials (carbon nanotubes and graphene oxide) to name a few. Targeting is achieved by NP surface conjugation with ligands (peptides, scFv, antibodies, affibodies, aptamers, etc.) that generally are designed to bind to receptors over-expressed on the target cell leading to receptor-mediated endocytosis (Davis et al., 2008). Depending on the drug cargo, this general endosomal delivery strategy could have some limitations. For example, endosomal escape of the cargo is needed to avoid degradation and exosome expulsion, additionally and importantly, to optimize therapeutic efficacy it might be necessary to direct certain cargos (e.g. proteins, siRNA, small molecules etc.) to specific organelles (e.g. ribosome, endoplasmic reticulum, or nucleus) as opposed to the cytosol (Delehanty et al., 2010; Paulo et al., 2011). While modification of particles with fusogenic peptides or cationic polymers aids endosomal escape into the cytosol through, e.g. osmotic swelling and disruption, and cargo can be conjugated with trafficking ligands such as nuclear localization sequences (Ashley et al., 2011), the use of iPhage identified peptides to accomplish both internalization and intracellular delivery could greatly simplify and improve upon current approaches.

In this regard an excellent potential nanocarrier platform for peptide display is the ‘protocell’ (Ashley et al., 2011; Ashley et al., 2012; Epler et al., 2012). Targeted protocells are formed by fusion of supported lipid bilayer membranes (similar to liposomes) on high surface area ($>1000 \text{ m}^2/\text{g}$) mesoporous silica nanoparticle cores (50–200 nm in diameter) followed by conjugation with targeting (and optionally trafficking ligands and PEG). They synergistically combine the advantages of liposomes (low inherent toxicity, immunogenicity, and long circulation times) and porous nanoparticles (stability and an enormous capacity for multiple cargos and disparate cargo combinations). Important to the concept of a peptide display platform, we have demonstrated that protocell-supported lipid bilayer (SLB) membranes retain both high in-plane, two-dimensional mobility and high stability against destabilization on exposure to blood components without leakage of drug cargos from the silica core. (Ashley et al., 2011) High in-plane mobility enables protocells, incorporating very low densities of targeting peptide ligands in the SLB, to bind selectively to target cells via multivalent binding enabled by *targeting ligand diffusivity* and recruitment by cell surface receptors. The low peptide density in turn allows high affinity, cell-specific binding while minimizing off-target binding and immunogenicity. For the case of display of peptides identified by iPhage, the fluid SLB display platform is anticipated to allow peptides to assemble from low density into domains reconstituting the contextual multivalency of the PIII phage selection library used to identify the phage, while at the same time avoiding immunogenicity stimulated by densely repeating patterns of peptides. To enable selective binding, internalization, and intracellular targeting, we suggest further, secondary conjugation of the protocell with a ligand that selectively binds a non-internalized receptor. iPhage identified peptides would then result in internalization and directed intracellular delivery to the target organelle.

Time considerations—The protocol detailed in this unit may be performed in ~8 weeks by investigators with a basic skill set in biochemistry, molecular, and cell biology.

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Figure 1.

Cloning strategy to generate the iPhage library. The f88-4 phage vector contains two capsid genes encoding a wild-type (wt) protein VIII (pVIII) and a recombinant protein VIII (rpVIII). The recombinant gene VIII contains a foreign DNA insert with a *HindIII* and a *PstI* cloning site. The *tac* promoter controls the expression of the rpVIII. Annealed oligonucleotides encoding the penetratin (pen) peptide are cloned in frame with the rpVIII (f88-4). Next, the fUSE5 and f88-4/pen genomes are fused to produce the iPhage display vector. The PCR-insert library is cloned into the *SfiI* endonuclease site. Representation of

the assembled phage particle expressing the wt major coat protein pVIII (gray), rpVIII-pen (green); rpIII, minor coat protein (red-square); TetR, tetracycline resistance gene (white).

Figure 2.

Phage vector (f88-4, fUSE5, and iPhage) purification by CsCl. For maximum phage plasmid purity, perform a CsCl/EtBr gradient. After ultracentrifugation, the lower plasmid band (dsDNA band) is recovered and is precipitated by addition of isoamyl alcohol into the DNA mix.

Figure 3.

Synchronous selection of a random peptide iPhage library. KS1767 cells are incubated with the random peptide iPhage library for 24hr at 37°C. The following day, cells are washed with PBS and subsequently detached with trypsin. Cells are incubated with hypotonic buffer, and placed in a standard Dounce homogenizer to disrupt cell membranes. The mitochondria/endoplasmic reticulum (ER) fraction is obtained by differential centrifugation at 4°C. The subcellular fraction-bound phage population is recovered through infection of log-phase K91/kan *E. coli*. Phage is purified by PEG/NaCl precipitation and prepared for the second round of selection. This process is performed as many times as needed. Usually two to four rounds of selection are enough to isolate enriched iPhage particles.

Figure 4. Flowchart of iPhage library data mining. Peptide sequences recovered from iPhage screening are submitted to vigorous bioinformatic analysis.