

MRBLE-pep Measurements Reveal Accurate Binding Affinities for B56, a PP2A Regulatory Subunit

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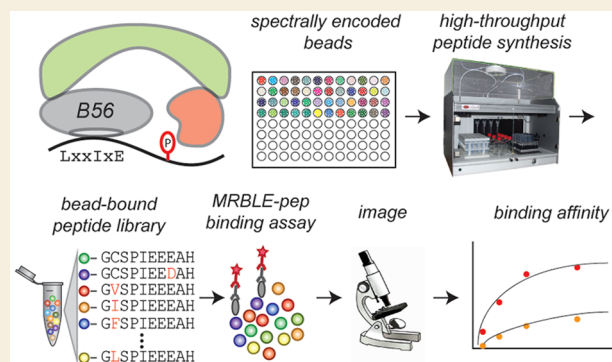
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ABSTRACT: Signal transduction pathways rely on dynamic interactions between protein globular domains and short linear motifs (SLiMs). The weak affinities of these interactions are essential to allow fast rewiring of signaling pathways and downstream responses but also pose technical challenges for interaction detection and measurement. We recently developed a technique (MRBLE-pep) that leverages spectrally encoded hydrogel beads to measure binding affinities between a single protein of interest and 48 different peptide sequences in a single small volume. In prior work, we applied it to map the binding specificity landscape between calcineurin and the PxIxIT SLiM (Nguyen, H. Q. et al. *Elife* 2019, 8). Here, using peptide sequences known to bind the PP2A regulatory subunit B56 α , we systematically compare affinities measured by MRBLE-pep or isothermal calorimetry (ITC) and confirm that MRBLE-pep accurately quantifies relative affinity over a wide dynamic range while using a fraction of the material required for traditional methods such as ITC.

KEYWORDS: MRBLE-pep, B56, PP2A, binding affinities, signal transduction



INTRODUCTION

Cellular signaling transduction pathways are essential for every living organism. At the molecular level, many critical interactions within these pathways involve a globular protein domain binding a short (3–10 amino acid) linear motif (SLiM) within an intrinsically disordered region of a target protein.^{2–4} These protein–protein interactions (PPIs) are considered attractive therapeutic targets, and there has been significant recent interest in developing small molecules to specifically target these PPI domains (e.g., PROTACS).⁵ However, many protein–SLiM interactions remain either uncharacterized or poorly characterized, as they tend to be relatively weak (K_d values in the 1–10 μ M range) and are often dynamically regulated via reversible post-translational modifications that modulate binding affinity to alter downstream responses, which complicates their detection, measurement, and characterization. New technologies for discovering, validating, and characterizing protein–SLiM interactions are therefore essential to unlock their therapeutic potential.

High-throughput technologies such as affinity purification coupled to mass spectrometry,⁶ phage display,^{7,8} and yeast two-hybrid assays,⁹ among others, have dramatically enhanced the pace of candidate protein–SLiM interaction discovery. Furthermore, computational modeling of motif determinants can allow *in silico* screening of entire proteomes to determine motif candidates.¹⁰ However, these high-throughput methods generate large numbers (100s–1000s) of candidate interactors,

many of which may be false positives. Validating and quantifying these low affinity binding interactions remain an experimental bottleneck, typically requiring sample- and labor-intensive low-throughput techniques such as isothermal calorimetry (ITC), surface plasmon resonance (SPR), and fluorescence polarization anisotropy, all of which require large amounts of purified protein.

In recent years, several technologies have attempted to bridge this gap between high-throughput, qualitative assays, and low-throughput, quantitative assays. Peptide microarrays¹¹ and Luminex bead-based assays¹² allow multiplexed measurements of ~10–1000 interactions in parallel, but do not take place at equilibrium and therefore cannot return thermodynamic binding constants. Microscale thermophoresis and hold-up assay approaches can facilitate such thermodynamic measurements and have successfully been applied toward a variety of systems, including PDZ- and chromo domain peptide interactions, antibody/antigen interactions, and receptor/ligand interactions.^{13–15} However, they still require fairly high amounts of purified protein.

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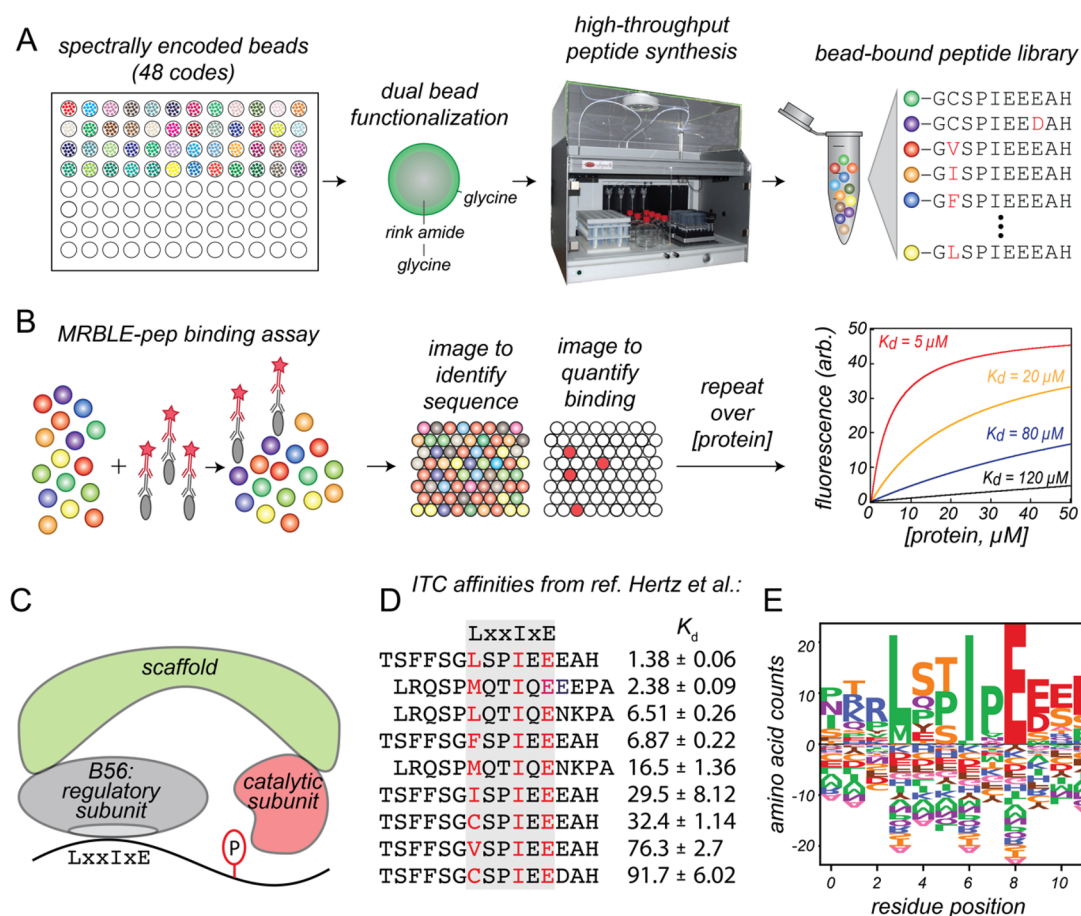


Figure 1. Overview of MRBLE-pep workflow and B56 specificity. (A) Schematic showing MRBLE-pep peptide library production. Spectrally encoded hydrogel beads containing unique ratios of lanthanide nanophosphors (LNPs) are functionalized with rink amide/glycine linkers within bead cores and glycine linkers in the outer shell; after functionalization, peptides are synthesized directly on beads with a 1:1 linkage between the peptide sequence identity and embedded spectral code via solid-phase peptide synthesis in a high-throughput peptide synthesizer. (B) MRBLE-bound peptide libraries are then pooled and incubated with a protein of interest, primary antibody, and labeled secondary antibody. After binding reactions reach the steady state, beads are imaged in the lanthanide channels (to identify the peptide sequence associated with each bead) and in fluorescence channels (to quantify the amount of bead-bound protein). Measurements of binding at multiple concentrations can be globally fit to Langmuir isotherms to quantify interaction affinities. (C) Cartoon model of the heterotrimeric B56-PP2A complex binding a substrate containing the LxxIxE SLiM recognition site. (D) Affinities measured by ITC for a set of nine peptides containing systematic substitutions within the LxxIxE SLiMs from Kif4A and FoxO3 (data from Hertz et al. 2016). (E) Consensus position-specific specificity matrix generated from known B56 binding sites (data from <http://slim.icr.ac.uk/pp2a/index.php?page=instances#28884018> and ^{6,22} logo generated using LogoMaker software.³²)

To complement these approaches, we previously developed MRBLE-pep, a technology that leverages spectrally encoded beads for medium-throughput quantitative measurement of protein–peptide interactions using very small amounts of material (2 μmol in 200 μL for one concentration experiment, or ~ 25 μg for B56 α , the protein analyzed here). To create spectrally encoded beads, we use a microfluidic device to generate porous PEG-DA hydrogel droplets containing specific ratios of four different species of lanthanide nanophosphors (LNPs), each of which comprises a unique spectral code (MRBLEs, for microspheres with ratiometric barcode lanthanide encoding)^{16,17} Using a custom-built fraction collector,¹⁸ we then direct all beads containing a given code to a specified well within a standard multiwell plate (Figure 1A). After collection, beads are chemically functionalized with Fmoc–glycine and then transferred to a high-throughput peptide synthesizer for Fmoc solid phase peptide synthesis, yielding MRBLE-pep libraries with a unique 1:1 linkage between a peptide sequence and embedded spectral code (Figure 1A). After synthesis, MRBLE-pep bead libraries are

pooled and incubated with a fluorescently labeled protein of interest, followed by imaging to quantify protein binding and identify embedded codes (and thus, the underlying peptide sequence) (Figure 1B). By repeating MRBLE-pep measurements over multiple protein concentrations, we generate concentration-dependent binding curves, which are fitted to Langmuir isotherms to quantify interaction affinities.

In prior work, we applied MRBLE-pep to probe binding interactions between human calcineurin (CN), the Ca²⁺/calmodulin regulated phosphatase and target of immunosuppressant drugs,¹⁹ and systematic mutations within the known PxIxIT SLiM. CN affinities for six PxIxIT peptide sequences correlated with those previously measured via orthogonal techniques and successfully predicted the strength of signaling inhibition in cells.¹ However, these affinity comparisons were limited and included several peptides with widely varying affinities reported in the literature. Unfortunately, our own attempts to acquire comparable data for these peptides using established technologies were unsuccessful.

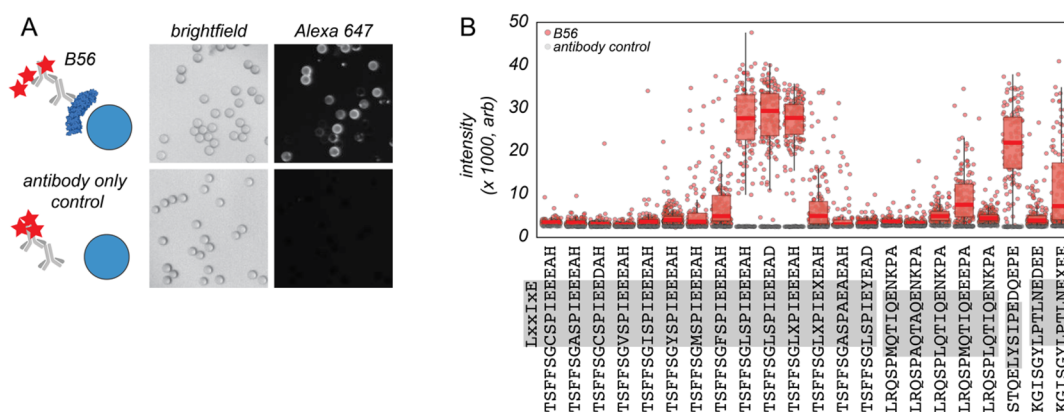


Figure 2. Example images and measured fluorescence intensities for beads incubated with antibodies and B56 or antibodies alone (negative control). (A) Schematic of binding assays and example images of beads in the bright field and Alexa 647 fluorescence channels. (B) Median binding intensities for each bead (markers) and 95% confidence intervals (box plots) for binding assays with B56 and antibodies (red) or antibodies alone (gray) for a MRBLE-pep assay with 22 different peptide sequences (X indicates phosphorylated serine).

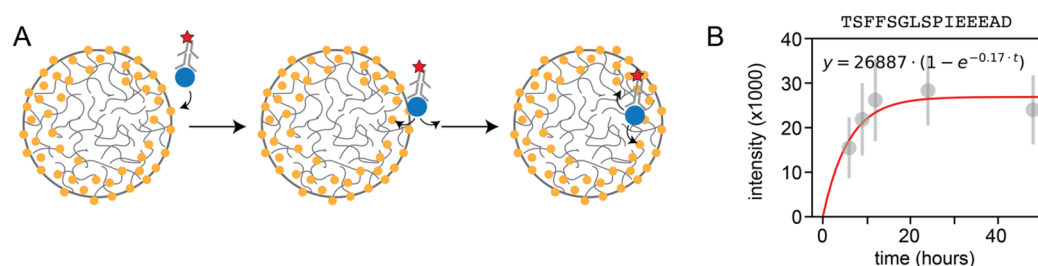


Figure 3. Hydrogel beads reduce dissociation rates to allow equilibrium binding measurements for even weak interactions. (A) Cartoon schematic showing proposed mechanism in which proteins binding to bead-bound peptides become trapped in bead hydrogel mesh. (B) Time course showing measured intensity as a function of time for B56-antibody complexes interacting with a peptide displayed by MRBLE hydrogel beads. Markers indicate median intensities, error bars indicate standard deviation, and red line indicates a fit to kinetic binding model (equation and fitted parameters displayed at top of graph).

Here, we extend the capabilities of the MRBLE-pep assay and further validate MRBLE-pep-derived affinity measurements using B56 α , a regulatory subunit of protein phosphatase 2A (Figure 1C).^{20,21} In recent work, the Nilsson group and others established that PP2A-B56 α substrate specificity relies on a protein–protein interaction between B56 α and the LxxIxE SLiM found in various substrates including KIF4A (a kinesin motor protein important for chromosome segregation) and FOXO3 (a transcription factor important for processes such as apoptosis and autophagy) (Figure 1D).^{6,22} As differences in affinity between B56 α and different substrates are thought to dictate the strength and relative timing of downstream signaling responses, the authors quantified the effects of multiple amino acid substitutions on binding affinities (K_d s) for eight different LxxIxE SLiM peptide scaffolds, revealing that substrate recognition by B56 α depends on additional amino acids beyond the conserved L, I, and E residues. This set of 50 affinities with a wide dynamic range (spanning from ~ 1 – $100 \mu\text{M}$), all measured by a single researcher expert in ITC, provide an ideal comparison set for testing the ability of MRBLE-pep to accurately return affinity information. By directly comparing MRBLE-pep- and ITC-derived affinities for B56 α protein interacting with nine distinct peptides, we demonstrate that the MRBLE-pep measurements are in good agreement with ITC literature data. Importantly, the rank order of affinities between different peptides is preserved in MRBLE-pep measurements resulting in the same conclusions in terms of the binding sequence variations while requiring significantly less material.

RESULTS

To test if MRBLE-pep can return accurate affinity information for an additional PPI system that also requires a different mode of protein detection, we measured binding of purified untagged B56 α protein (graciously provided by the Nilsson lab⁶) to two libraries of peptides (containing 22 and 24 peptides, respectively) via MRBLE-pep. To facilitate direct comparison between MRBLE-pep and ITC measurements over a wide dynamic range, each library included nine peptides with previously measured affinities for B56 α spanning 1 to 92 μM (Figure 1D).

A Generalizable Method for Fluorescent Detection of MRBLE-Bound Protein

Unlike in the original MRBLE-pep publication¹ where we detected binding of His-tagged CN to libraries of MRBLE-bound peptides via binding of a fluorescently labeled anti-His antibody, here we detected binding of untagged B56 α protein using a commercially available anti-B56 α primary antibody and an Alexa-647-labeled secondary antibody (Figure 2A,B). Fluorescence images of bead-bound protein revealed a strong bead-associated Alexa-647 signal only in the presence of the B56 α protein, establishing that nonspecific antibody binding to MRBLE-bound peptides was insignificant (Figure 2A,B). Fluorescent signals for beads coupled to known peptides with high affinity for B56 α were >30-fold higher than those for negative controls, establishing that binding was detected with a high dynamic range (Figure 2A,B).

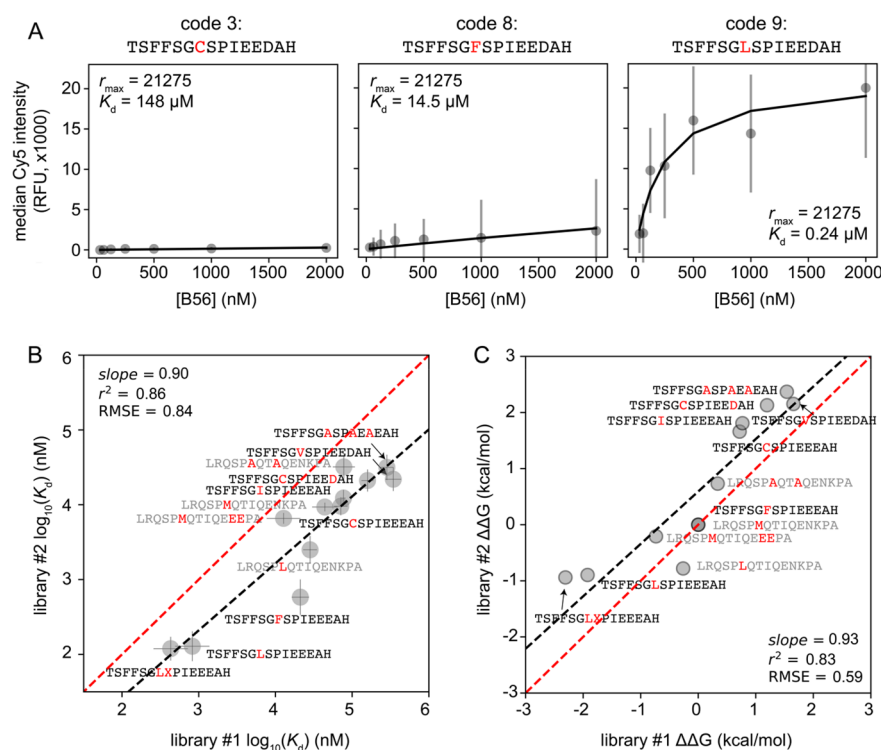


Figure 4. Technical reproducibility of MRBLE-pep replicate binding measurements. (A) Concentration-dependent binding data from library #1 for B56 interacting with three peptides containing different single amino acid substitutions within the Kif4A LxxIxE SLiM. Gray markers indicate median fluorescence intensities for all MRBLEs displaying a particular peptide sequence at a given concentration; error bars indicate standard deviation of intensities. Solid gray lines denote Langmuir isotherm fits sharing a single maximum intensity saturation value (see methods). (B) Measured \log_{10} -transformed binding affinities for 12 peptides measured via two independent full technical MRBLE-pep replicate experiments. Gray markers indicate the mean of three independent MRBLE-pep replicates for a given library; error bars denote the associated standard deviation. The red dashed line indicates a 1:1 relationship and the black dashed line indicates the results of a linear regression to the \log_{10} -transformed K_d values. (C) Measured $\Delta\Delta G$ values for 12 peptides measured across two full technical MRBLE-pep replicates. Gray markers indicate the mean of three independent MRBLE-pep replicates for a given library; error bars denote the associated standard deviation. The red dashed line indicates a 1:1 relationship, and the black dashed line indicates the results of a linear regression (X indicates phosphorylated serine).

Estimating Time-to-Equilibrium and Verifying Appropriate Concentrations

Accurately estimating interaction affinities by fitting Langmuir isotherms to observations of concentration-dependent binding relies on two main assumptions. First, interactions must have reached steady state, with no further change in the relative bound and unbound protein fractions over time. Second, the amount of protein ligand available for binding must be in vast excess of the amount of MRBLE-bound peptides in each experiment and significantly higher than expected interaction K_d s.²³ For protein-peptide interactions (PPIs) in solution, the time required for a given PPI to reach equilibrium (K_{eq}) can be loosely approximated by the dissociation rate of the interaction (k_{off}). For relatively weak interactions (K_d s in the 1–10 μM range), this dissociation rate typically ranges from $\sim 10^3$ to 10^5 s, corresponding to a time to reach equilibrium of ~ 0.04 –4 s.²⁴ In our prior MRBLE-pep experiments, the time required for CN-peptide interactions to reach equilibrium was approximately 14 h, presumably because CN proteins interacting with peptides covalently coupled to the hydrogel matrix were sterically constrained within the porous hydrogel mesh, where the high local concentration of peptide ligands promotes rebinding (Figure 3A). These slow equilibration times are critical for assay performance, as they make it possible to wash and image beads without loss of weakly bound material during imaging. However, it remained unclear if additional proteins would also require extended times to reach assay equilibrium

or whether this property would depend on additional factors such as peptide density, buffer composition, or the identity of the protein itself.

To experimentally test the time required for MRBLE-pep B56 α binding to reach equilibrium, we incubated the B56 α protein at 2 μM with a MRBLE-bound peptide sequence with high affinity for B56 α (TSFFSGLSPIIEEAD, code 10) (Figures S1–S4). The reactions required incubation for ~ 24 h at 4 $^\circ\text{C}$ to reach equilibrium (Figure 3C, Figures S1–S4), providing additional support for a model in which protein/peptide dissociation rates are significantly slowed by protein rebinding to other peptides immobilized within the hydrogel matrix. On the basis of these measurements, we quantified binding for all subsequent B56 MRBLE-pep experiments after 24 h.

To confirm that B56 α protein was indeed in vast excess, we first calculated the expected concentrations of B56 α protein and available peptides within each binding assay. For a given peptide sequence, we use approximately 100 beads with an estimated peptides density of 10^8 peptides per bead.¹ We therefore have a peptide concentration of 2.4×10^{11} peptides in a 100 μL assay volume for 24 different codes and a protein concentration of 1.86×10^{13} at the lowest concentration of protein (31 nmol). For direct experimental validation, we additionally removed the supernatant after 24 h of incubation and quantified the remaining B56 α via Western blot,

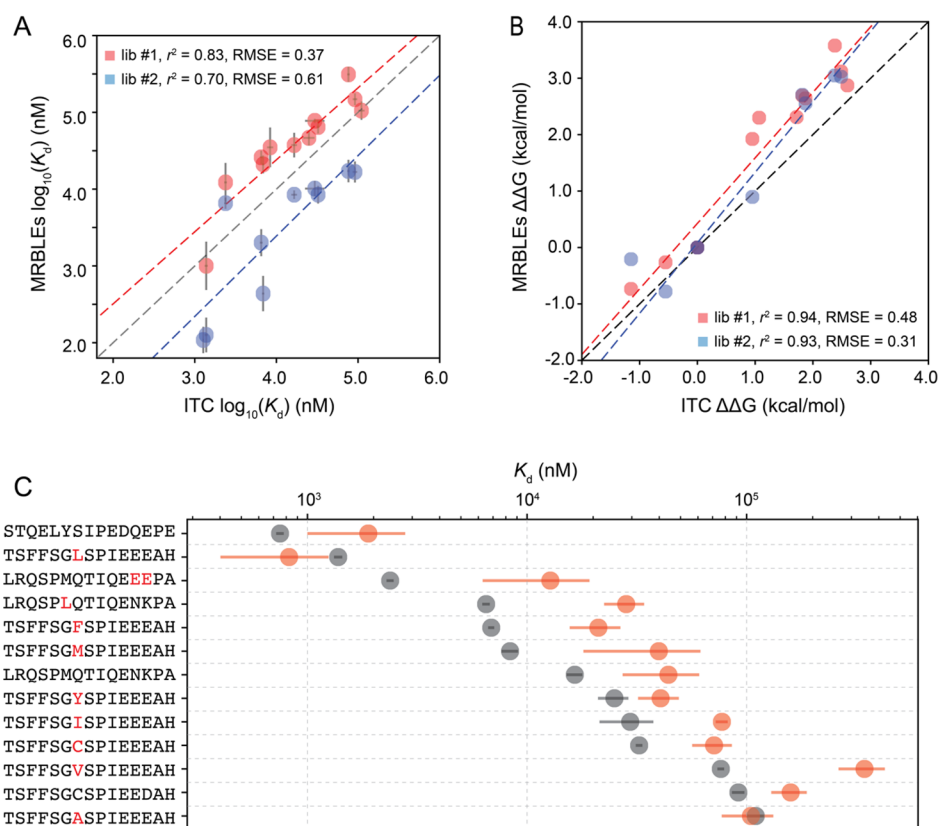


Figure 5. Comparison between MRBLE-pep and ITC affinity measurements. (A) Comparison of absolute affinities (K_d s) measured via MRBLE-pep libraries #1 (red) and #2 (blue) and ITC (data from Hertz*,Kruse*, et al.) for nine peptides derived from Kif4A and FoxO3 SLiM recognition motifs present in all three libraries (see Figure 1D). Each MRBLE-pep measurement represents the median of three technical replicates, and error bars indicate the standard error of the mean; for ITC measurements, error bars indicate the uncertainties returned from Langmuir isotherm fits. (B) Comparison of relative differences in affinity ($\Delta\Delta G$ s) measured via MRBLE-pep libraries #1 (red) and #2 (blue) and ITC (data from Hertz*,Kruse*, et al.) for nine peptides derived from Kif4A and FoxO3 SLiM recognition motifs present in all three libraries (see Figure 1D). (C) Comparison between affinities measured by MRBLE-pep library #1 (red) and ITC (data from⁶) for 13 peptides present in both libraries.

establishing no depletion over the full range of concentrations tested (Figure S5).

Comparing Affinities Measured across MRBLE-pep Technical Replicates

Next, for each MRBLE-pep library, we performed three technical replicate experiments measuring concentration-dependent binding for B56 α protein at seven concentrations ranging from 31 to 2000 nmol, then globally fit the median intensities for all beads containing a given code at each concentration to a Langmuir isotherm (Figure 4A, Figures S6–S13):

$$I_i = \frac{I_{\max} * [B56]}{[B56] + K_{d,i}} \quad (1)$$

Here, I_i represents the median fluorescence signal associated with all beads bearing a particular peptide at a given concentration, I_{\max} is a global saturation value shared across all peptides, $[B56]$ represents the soluble B56 α concentration available for binding, and $K_{d,i}$ is the binding affinity for a given peptide. By constraining all curves to share a single saturation value for fluorescence intensity, this global fitting procedure returns reliable binding affinity estimates even for peptides with K_d values well above the maximum B56 α concentration used in the assay (see Materials and Methods). We then convert measured K_d values (absolute affinities) to relative

differences in binding energies upon mutation ($\Delta\Delta G$ s) using the following equation:

$$\Delta\Delta G_i = RT \ln(K_{d,i}/K_{d,\text{ref}}) \quad (2)$$

Here, $\Delta\Delta G_i$ represents the relative change in binding energy for a given peptide with $K_{d,i}$ relative to a “reference” peptide. R and T represent the natural gas constant and temperature, respectively. Pairwise comparisons of measured K_d and $\Delta\Delta G$ values between replicates for both libraries established that measurements were highly reproducible, with r^2 values of 0.73–0.99 and RMSEs of 0.11–0.37 for K_d values ranging over nearly 3 orders of magnitude and r^2 values of 0.95–0.98 and RMSEs of 0.03–0.65 for $\Delta\Delta G$ values spanning nearly 5 kcal/mol (Figures S9 and S13). Measurements for the same peptide across the two different MRBLE-pep libraries also showed strong agreement ($r^2 = 0.86$ and RMSE = 0.84 for K_d comparisons) (Figure 4B). Overall, measured affinities for peptides from library #2 were systematically higher than for library #1, consistent with prior observations that uncertainties in the estimated intensity at saturation can lead to systematic variations in measured affinities. However, relative differences in binding energies remained consistent, particularly when considering systematic mutations within a given peptide backbone ($r^2 = 0.83$ and RMSE = 0.59 for $\Delta\Delta G$ comparisons, corresponding to <2-fold differences across experiments).

Comparing Affinities Measured via MRBLE-pep and ITC

Next, we sought to directly compare affinities measured via MRBLE-pep and ITC (isothermal calorimetry). While ITC has traditionally been considered the “gold standard” method for measuring K_{d} s for protein interactions, these experiments require large amounts of highly purified protein, particularly for weak protein–peptide interactions (900 μg per protein/peptide interaction for K_{d} s in the low micromolar range). MRBLE-pep measurements require significantly less material (25 μg for 22 protein/peptide interaction measurements, or 1 μg per interaction) and labor (as multiple protein/peptide interactions can be assessed in a single experiment), but to date have only been shown to return thermodynamic binding information for a single protein (CN).

Measured affinities (K_{d} s) for B56 α binding to 12 peptides containing systematic substitutions within the LxxIxE motif found within Kif4A or FoxO3 showed strong correlation between the two methods ($r^2 = 0.83$ and 0.70 for libraries 1 and 2, respectively) (Figure 5A). Measured affinities from library #1 showed better agreement with ITC-derived affinities (RMSE = 0.37 and 0.61 for libraries 1 and 2, respectively), largely due to a single measurement for a FoxO3 variant containing additional negatively charged residues at the C-terminal end of the sequence (LRQSPMTIQEEPPA) (Figure 5B, Table S1). Overall, library 2 measurements returned systematically higher affinities, potentially due to small variations in the surface peptide density of displayed peptides between replicates. However, measurements of relative changes in binding affinities upon mutation ($\Delta\Delta\text{G}$ s) showed strong correlation for both libraries ($r^2 = 0.93$ and 0.94 for both libraries) with RMSEs of 0.48 and 0.31, respectively, and the rank order of preferred sequences remained largely unchanged. Together, these comparisons establish that MRBLE-pep returns binding affinities with similar accuracy to ITC but using 1/500th of the material.

Applying MRBLE-pep to Investigate How Mutations within LxxIxE SLiMs Affect B56 Binding

Having established the accuracy of MRBLE-pep, we applied it to investigate the effects of systematic substitutions within the LxxIxE SLiM motifs present within Kif4A, FoxO3, GEF-H1, and Rac-GAP1. In particular, we investigated the influence of different amino acids in the first position and those surrounding the acidic last position of the motif. In addition, we probed how phosphorylation at different positions in close proximity to key positions affected binding affinities. (Figure 6). Within Kif4A, the LxxIxE motif appears as a CxxIxE sequence (Figure 6A). As expected, making a C to L substitution at the first position within the SLiM (which alters the site to look more like the known consensus) enhances affinity by ~ 2.5 kcal/mol (Figure 6A). Overall, the L residue is strongly preferred at this position, with F and M substitutions also moderately enhancing binding relative to the native C. In the presence of mutations to this first position, mutating the additional I and E conserved residues reduces binding to background levels. Surprisingly, substituting the L or the E position with a Y in a high affinity LxxIxE motif did not completely abolish binding (reduction of ~ 2 or ~ 3 kcal/mol, respectively), suggesting that SLiMs containing an L in this first position or multiple acidic residues at the C-terminal boundary may be able to tolerate substitutions at other conserved positions while maintaining biological function

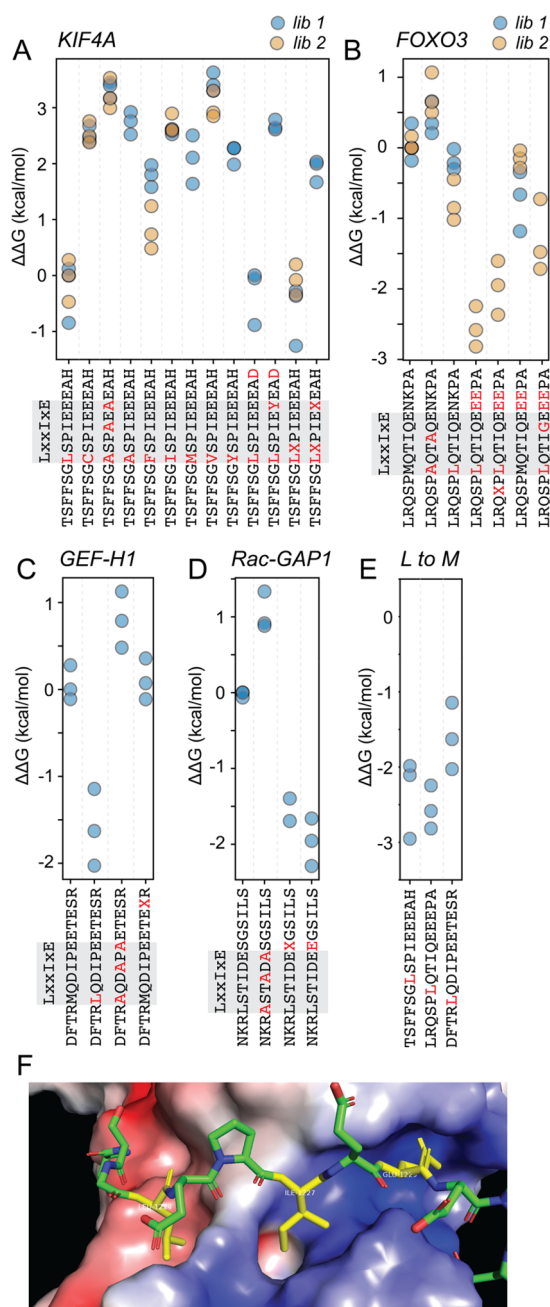


Figure 6. MRBLE-pep measurements quantifying the effects of systematic amino acid substitutions within the Kif4A, FoxO3, GEF-H1, and Rac-GAP1 LxxIxE SLiM on B56 binding affinity (X indicates phosphorylated serine). (A) Measured $\Delta\Delta\text{G}$ s for systematic substitutions within the Kif4A LxxIxE SLiM relative to the median TSFFSGLSPIIEEAAH value. (B) Measured $\Delta\Delta\text{G}$ s for systematic substitutions within the FoxO3 LxxIxE SLiM relative to the median WT (LRQSPMTIQENKPA) value. (C) Measured $\Delta\Delta\text{G}$ s for systematic substitutions within the GEF-H1 LxxIxE SLiM relative to the median WT (LDFTRMQDIPEETESR) value. (D) Measured $\Delta\Delta\text{G}$ s for systematic substitutions within the Rac-GAP1 (NKRLSTIDESGILLS) value. (E) Measured $\Delta\Delta\text{G}$ s for M to L substitutions within three different peptide backbones (Kif4A, FoxO3, and GEF-H1). (F) View of the B56-Kif4A binding interface from ref 25 with the B56 surface colored by local charge density (PDB: 6OYL, generated with PyMOL).

(Figure 6A). However, future *in vivo* work is required to test this hypothesis.

In recent work, the Nilsson lab also reported that phosphorylation generally increases B56 α binding affinities when adjacent to either the glutamic acid residues at the C-terminus of the LxxIxE SLiM or to the conserved N-terminal leucine. Here, we find that the effects of phosphorylation are strongly context-dependent: while a single phosphorylated residue adjacent to the conserved leucine leads to slightly enhanced affinities, the addition of a second phosphorylated residue at the C-terminus of the Kif4A SLiM dramatically decreases binding (Figure 6A). By contrast, the addition of a phosphorylated serine N-terminal to the FoxO3 LxxIxE motif reduces binding (Figure 6B), phosphorylation of residues adjacent to the glutamic acids within the GEF-H1 SLiM has no effect on affinities, and phosphorylation immediately C-terminal to the Rac-GAP1 LxxIxE SLiM dramatically enhances binding (Figure 6C,D). Interestingly, an additional E after the obligatory E seems to increase affinity more than a phosphorylated serine (Figure 6D). Furthermore, we observed a strong decrease in binding affinity when mutating position five (LxxIxE) from D to G in FoxO3, indicating that this position could be important for fine-tuning the affinity between B56 α and its binding partners (Figure 6B); future experiments are required to systematically probe how substitutions at this position alter affinity. Together, these results highlight the importance of local sequence context in determining binding strength, an observation further supported by the fact that M to L substitutions within a variety of different peptide backbones have different effects (Figure 6E). In the previously published crystal structure for B56 bound to the Kif4A peptide,²⁵ the C-terminal conserved E residues binds near a strongly basic pocket, suggesting that additional C-terminal phosphorylated residues reduce binding via electrostatic repulsion (Figure 6F). However, other effects cannot be easily rationalized by inspecting the crystal structure. As was previously seen for CN binding to PxIxIT motif,¹ mutations to solvent-exposed residues can dramatically alter affinity, potentially by altering the conformational ensembles of unbound peptides. That the same substitution within different scaffolds can lead to opposite effects suggests that additional structures will be required to dissect mechanisms of B56 substrate specificity.

DISCUSSION

In prior work, we described the development of the MRBLE-pep assay and demonstrated that MRBLE-pep was able to quantitatively profile the binding specificity landscape for CN interacting with a library of ~400 peptides containing systematic substitutions within the PxIxIT SLiM. Here, we further validate that MRBLE-pep agrees with previous ITC measurements in reporting the relative effects of amino acid substitutions on binding affinities of an additional SLiM (LxxIxE) for its receptor (B56 α), while using a fraction of the required material and requiring a fraction of the time. Moreover, we establish that protein binding can be accurately quantified using an unlabeled primary antibody and fluorescently labeled secondary antibody, expanding the capabilities of the assay to examine proteins that are difficult to tag and/or purify.

While technical replicates using independent MRBLE-pep libraries showed systematic shifts in raw affinities (Figure 5), we establish that measured $\Delta\Delta G$ s and the rank order of peptide sequence preferences are highly reproducible. We speculate that these systematic shifts may result from

differences in the density of displayed peptides after on-bead synthesis. In recent work, we described a dramatically simplified method for encoded bead production and functionalization (MRBLEs 2.0),²⁶ and we anticipate that peptide synthesis on beads produced via this new pipeline will yield more consistent density coverage. Furthermore, by simplifying the technology we hope that these improvements will allow researchers to more broadly adopt MRBLE-pep to systematically characterize SLiM binding determinants and to validate candidate interactions identified by high-throughput phage-display, yeast-two-hybrid or *in silico* screens.

MATERIALS AND METHODS

MRBLE Production and Peptide Synthesis

We produced MRBLEs spectrally encoded hydrogel beads as described previously.^{1,27}

B56 Expression and Purification

B56 α was a kind gift from the lab of Jakob Nilsson and was expressed and purified as described previously.⁶

MRBLE-pep Time-to-Equilibrium Assays

To reduce nonspecific binding, we “blocked” beads by incubating approximately 500 beads per code with PBST (0.1% Tween-20) and 5% BSA for 1 h at RT. To allow labeled antibody to bind B56 protein prior to bead assays, we preincubated 2 μ mol recombinant B56 protein with 1 μ mol mouse anti-B56 antibody (Clone 23/B56 α BD biosciences, 610615) and 1 μ mol goat antimouse IgG Alexa Fluor 647 (Thermo Fisher A-21235) in 500 μ L binding buffer (50 mM Tris pH = 7.5, 150 mM NaCl, 0.1% TWEEN 20) for 1 h at 4 $^{\circ}$ C. Prior to beginning binding assays, we washed beads once with binding buffer and divided them into five 200 μ L Eppendorf tubes. After decanting the binding buffer, we added 100 μ L of the B56 α protein and anti-B56 and antimouse IgG antibody complex. At specified times, we removed protein-antibody complexes, washed the beads once with 100 μ L of binding buffer, and resuspended them in 20 μ L of PBST prior to imaging.

Western Blotting to Test for Depletion of B56 after Binding Assays

Each gel electrophoresis reaction used 6 μ L of unbound B56 α protein-antibody complex and 2 μ L of 4x SDS-PAGE sample buffer (Thermo Fisher). After heating the samples to 95 $^{\circ}$ C for 10 min, we loaded samples on a SDS-PAGE gel (4–20% Precast Protein Gels, Bio-Rad #4561095DC) and separated the protein at 100 V for 60 min. We then blotted the separated proteins onto a PVDF membrane for 90 min at 400 mA using a Bio-Rad wet blotting system. The membrane was blocked with 3% BSA in TBS-T for 30 min at room temperature with gentle agitation. We removed the buffer and added fresh TBS-T with 3% BSA and Goat anti-Mouse IR-Dye 700 (1:5000 diluted) to obtain a background signal from the Mouse anti-B56 antibody present in the samples. After 2 h incubation with gentle agitation at room temperature, we washed the blot three times for 5 min with TBS-T, dried, and scanned using the Odyssey CLx imaging system (Licor Biosciences). The membrane was incubated again with 3% BSA in TBS-T for 30 min at room temperature. The buffer was exchanged and 500 ng of Mouse anti-B56 antibody was added and incubated for 2 h at room temperature. The membrane was again washed three times for 5 min with TBS-T, and then we added fresh buffer containing 1:5000 diluted Goat anti-Mouse IR-Dye 700 and incubated for 1 h at room temperature. After an additional washing, the membrane was scanned using the Odyssey CLx imaging system.

MRBLE-pep Concentration-Dependent Binding Assays

Each concentration-dependent binding assay used approximately 700 beads per code. To begin the assay, we blocked beads with PBST (0.1% Tween-20) and 5% BSA for 1 h at room temperature (RT) to reduce nonspecific binding. During this blocking step, we also preincubated 200 μ L of 2 μ mol recombinant B56 protein with 1 μ mol

mouse anti-B56 antibody (Clone 23/B56 α BD biosciences, 610615) and 1 μ mol goat antimouse IgG Alexa Fluor 647 (Thermo Fisher A-21235) for 1 h at 4 degrees to form B56/antibody complexes. Next, we washed beads once with 700 μ L of binding buffer and then divided them into seven 200 μ L Eppendorf tubes to yield approximately 100 beads per code per assay. B56/antibody complexes were diluted from 2 μ mol to the appropriate assay concentration using binding buffer containing 10% glycerol and added to Eppendorf tubes containing beads to a final reaction volume of 100 μ L. After 24 h, we removed the unbound protein–antibody complexes, washed beads once with 100 μ L of binding buffer, and resuspended them in 20 μ L of PBST.

MRBLE-pep Imaging

We imaged all beads exactly as previously described.^{1,27,28} At the end of this analysis, we quantified a median intensity for all pixels associated with this bead annulus for each bead.

Global Fitting of Concentration-Dependent Binding Curves

To generate binding curves for each code, we first plotted the median of all median bead annulus fluorescence intensities as a function of soluble B56 α concentration. To determine the K_d values for each protein/peptide interaction, we then globally fit the data for all codes to a single-site binding model:^{29–31}

$$I_i[\text{B56}] = \frac{I_{\max} \times [\text{B56}]}{[\text{B56}] + K_{d,i}}$$

Here, $I_i[\text{B56}]$ denotes the measured bead-associated Cy5 intensity as a function of soluble B56 concentration for a given code, I_{\max} is a global constant shared across all codes corresponding to the maximum bead-associated Cy5 intensity at saturating [B56], [B56] denotes the concentration of soluble B56 available for binding, and $K_{d,i}$ denotes the dissociation constant (K_d) for a given B56-peptide complex. This global fitting procedure assumes that (1) the binding interaction is measured at steady-state, and (2) the stoichiometry of binding remains constant across all B56-peptide interactions (making it possible to estimate K_d values even for very weak interactions with K_d values greater than the maximum soluble B56 concentration probed in the assay).

To calculate $\Delta\Delta G$ values across a given experiment, we used the following formula:

$$\Delta\Delta G_i = RT \ln(K_{d,i}/K_{d,\text{ref}})$$

where R is the gas constant (1.987×10^{-3} kcal/(K·mol)), T is 298 K, and $K_{d,i}$ is the median of the returned K_d values from technical replicates of concentration-dependent binding experiments for a protein–peptide interaction of interest and $K_{d,\text{ref}}$ is the median of the returned K_d values from technical replicates of concentration-dependent binding experiments for a reference protein–peptide interaction.

Comparing MRBLE-pep Technical Replicates for a Given Library

To compare measurements between technical replicates using a given MRBLE-pep library, we performed pairwise comparisons between each replicate and evaluated concordance via a linear regression of (1) \log_{10} -transformed K_d values for each peptide returned from the global fitting procedure described above, and (2) calculated $\Delta\Delta G$ values relative to a single reference peptide.

Comparing MRBLE-pep Measurements Across Libraries

To compare measurements across MRBLE-pep libraries, we directly compared (1) the median \log_{10} -transformed K_d value from the three technical replicates of each library and (2) $\Delta\Delta G$ values calculated from these median K_d values. In each case, we again evaluated agreement via a linear regression.

Comparing MRBLE-pep Measurements with ITC

To compare measurements between MRBLE-pep and ITC, we again directly compared the median \log_{10} -transformed K_d value from the

three technical replicates of each library with prior measurements from the Nilsson lab.⁶

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmeasuresciau.1c00008>.

Mean K_d (nM) and mean $\Delta\Delta G$ (kcal/mol) for peptides calculated from triplicate measurements for both libraries; supplemental figures (PDF)

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Notes

The authors declare the following competing financial interest(s): P.M.F. previously co-authored a patent describing production of spectrally encoded beads via ratiometric barcode lanthanide encoding (WO 2014/031902 A2).

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