Peptide library synthesis on spectrally encoded beads for multiplexed protein/peptide bioassays

Huy Q. Nguyen[1,2], Kara Brower[*,1], Björn Harink[*1,2], Brian Baxter[2], Kurt S. Thorn[*,2] & Polly M. Fordyce[*,1]

[1]Stanford University, Stanford CA 94305
[2]University of California San Francisco, San Francisco CA 94158
[*]These authors contributed equally to this work

ABSTRACT

Protein-peptide interactions are essential for cellular responses. Despite their importance, these interactions remain largely uncharacterized due to experimental challenges associated with their measurement. Current techniques (*e.g.* surface plasmon resonance, fluorescence polarization, and isothermal calorimetry) either require large amounts of purified material or direct fluorescent labeling, making high-throughput measurements laborious and expensive. In this report, we present a new technology for measuring antibody-peptide interactions *in vitro* that leverages spectrally encoded beads for biological multiplexing. Specific peptide sequences are synthesized directly on encoded beads with a 1:1 relationship between peptide sequence and embedded code, thereby making it possible to track many peptide sequences throughout the course of an experiment within a single small volume. We demonstrate the potential of these bead-bound peptide libraries by: (1) creating a set of 46 peptides composed of 3 commonly used epitope tags (myc, FLAG, and HA) and single amino-acid scanning mutants; (2) incubating with a mixture of fluorescently-labeled anti-myc, anti-FLAG, and anti-HA antibodies; and (3) imaging these bead-bound libraries to simultaneously identify the embedded spectral code (and thus the sequence of the associated peptide) and quantify the amount of each antibody bound. To our knowledge, these data demonstrate the first customized peptide library synthesized directly on spectrally encoded beads. While the implementation of the technology provided here is a high-affinity antibody/protein interaction with a small code space, we believe this platform can be broadly applicable to any range of peptide screening applications, with the capability to multiplex into libraries of hundreds to thousands of peptides in a single assay.

Keywords: Microfluidics, microparticles, biological multiplexing, bead-based assay, spectral encoding, lanthanides, solid-phase peptide synthesis, peptide arrays

1. INTRODUCTION

Multiplexed bioassays allow measurement of hundreds to thousands of biological signals within a single experiment[1]. These assays have made major impacts in basic research and clinical medicine, enabling high-throughput DNA binding analysis, immunophenotyping, analyte screening, and sensitive pathogen detection, among other examples[1-9]. Bead-based multiplexing technologies offer several distinct advantages, including solution-phase kinetics, ease-of-use, sample conservation, and built-in assay replicates for increased accuracy[1,2,6,7]. Spectrally encoded bead assays, where each spectral signature (or 'color') is uniquely associated with a single analyte, are particularly attractive because they often make use of existing imaging platforms for decoding that are readily available in research labs and the clinic (*e.g.,* microscopy and flow cytometry)[1,6,8-15]. Spectral encoding has been most widely implemented using the Luminex xMap technology, which boasts upward of 500 spectral codes[12]. However, most existing spectral technologies are incompatible with typical organic synthesis reagents, precluding direct synthesis of peptides, small molecules, or other compounds onto encoded beads[16]. This lack of direct routes for customizable library generation has limited the field, especially in the area of peptide-binding assays.

High-throughput, systematic measurements of protein-peptide binding interactions are critical for understanding protein mechanisms of action, parsing the relative importance of functional domains within a protein, elucidating cellular interaction networks, and even designing better therapeutics[4]. Multiplexed bioassays that sweep a large peptide space have made considerable progress in assessing the full landscape of peptide binding interactions, but spatial microarrays that employ parallel chemical synthesis approaches (*e.g.,* SPOT arrays[17-19]) require large amounts of protein and are

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prohibitively expensive[17]. Bead-based technologies can overcome this cost issue by acting as typical solid phase supports for peptide and protein synthesis, thereby requiring overall less reagent since coupling can take place with small solution volumes[10,11]. Ideally, a technology to fill this gap would allow the generation of custom peptide libraries by direct on-bead synthesis using an easily interpretable optical readout, enabling low-cost, high-throughput peptide library screening with flexibility for library iteration.

In this proceeding, we demonstrate the ability to generate customizable peptide libraries on spectrally encoded beads that can be used for a variety of binding and enrichment assays. We first use a previously developed microfluidic platform[20,21] to generate libraries of spectrally encoded beads containing multiple distinct ratios of lanthanide nanophosphors, each of which creates a unique 'color' (MRBLEs, or Microspheres with Ratiometric Barcode Lanthanide Encoding[20]). After bead generation, we assign a desired peptide sequence to each 'color,' amine functionalize the beads via Michael addition[22], and, using a peptide synthesizer, couple the assigned peptide sequence to all beads containing a particular 'color.' In this way, many different peptide sequences can be mixed together and tracked throughout the course of an experiment by simply the 'color' of their associated beads. An important feature of lanthanide-based encoding is that lanthanides are excited using deep UV light, preserving fluorescence channels for orthogonal detection of proteins interacting with bead-bound peptides using typical organic dyes. Here, we take advantage of this feature by demonstrating the ability to simultaneously read bead 'colors' and detect binding of up to three different fluorescently-labeled antibodies interacting with 46 different peptides in a single experiment. We anticipate that this technology could become broadly applicable as a means for assessing many molecular interactions in parallel in a conserved sample volume with the advantage of flexible, programmable one-bead, one-compound library generation directly on-bead.

2. METHODS

2.1 Encoded Bead Code Generation

Encoded beads are synthesized as reported previously[20,21]. Briefly, a custom multilayer microfluidic device allows for precise ratiometric incorporation of many distinct combinations of lanthanide nanophosphors (synthesized in-house[20]) within PEG diacrylate (Sigma) hydrogel droplets containing a UV-activatable crosslinker (LAP, synthesized in-house[20]). These droplets are then polymerized into microspheres by flowing them through a channel exposed to UV-B light and collected off-chip for further downstream assays.

2.2 Encoded Bead Fraction Collection

For downstream one-code, one-bead peptide library generation, a custom microfluidic fraction collector was built using an OEM ASI stage recovered from an Illumina GAIIx sequencer to collect all beads belonging to a single code as they exit the microfluidic device (~10,000 beads). Outlet PEEK outlet tubing (ID = 255 μm, 330 mm length) was connected to the microfluidic device and attached to a stationary positioning arm above the fraction collector stage. Multiwell (96 well) filter plates (Millipore) or solid phase reaction vessels (Applied Separations Inc.) were positioned in the fraction collector stage and aligned below the arm. An automated script positioned the stationary arm containing the output tubing above the relevant well for each code. A 5 minute oil wash was conducted following each code synthesis step to clear the output tubing for the next code.

2.2 Bead Buffer Exchange and Resuspension

Encoded beads in their collection vessels (*e.g.* solid phase reaction vessels or 96 well filter plates) were washed to eliminate mineral oil by sequentially rinsing with 5.0 % PBS SDS (3 x 5 mL)**,** 1.0% PBS TWEEN-20 (1.0% PBST, 3 x 5 mL), and 0.1% PBS TWEEN-20 (0.1% PBST, 3 x 5 mL) in a manifold (Promega) under vacuum aspiration. Buffer-exchanged beads were subsequently rinsed liberally with DMF (Sigma), and suspended in DMF (5 mL) for 1 hour before conjugation.

2.3 Cysteamine functionalization via Michael Addition

Encoded PEG diacrylate hydrogel beads (0.320 mmol/g) were incubated with cysteamine hydrochloride (20 eq) and pyridine (20 eq) in a mixture of H_2O:DMF (1:3), to a reaction volume of 0.50 mL/tube. The suspension was shaken on a plate rotator on high at ambient temperature overnight (16 hours). After incubation, the encoded beads were washed liberally with DMF, MeOH, DCM, and DMF (~ 2.0 mL each). Successful amine functionalization was checked via Kaiser test. All reagents were purchased from Sigma Aldrich.

2.4 FMOC amine-loading quantification and visualization

Encoded beads were assessed for amine-loading post-functionalization via a previously published protocol[23]. Briefly, encoded beads were incubated in DMF to a final volume of 600 µL with an FMOC protected amino acid, FMOC-Gly-OH (10.0 eq) and DIC (10.0 eq) for 3 hours at ambient temperature to couple the amino acid to the active amine group on the beads. Beads were then washed liberally with DMF, methanol, and DCM and allowed to dry under vacuum for 3 hrs. Subsequently a dry weight was taken for FMOC quantification in 10 mL volumetric flask. 2% DBU in DMF was loaded to 2 mL into the flask for FMOC deprotection and the reaction was allowed to proceed for 30 minutes under gentle rotation. FMOC fluorescence in the supernatant was subsequently assessed by a cuvette reader. As an additional visual confirmation of FMOC conjugation before deprotection, beads were imaged before and after FMOC-protected amino acid addition in a custom bead reactor microfluidic device under deep UV excitation (292 nm) and a filter for FMOC emission (435/40 nm). Images were taken for 2s at 2x binning under a 4x objective with an emCCD camera (Andor iXon).

2.5 On-bead peptide synthesis

Peptide synthesis on amine-functionalized encoded beads was performed using a Biotage Syro II peptide synthesizer in 20 µm fritted reaction vessels (Biotage), sequestered by code. Each code was assigned a known peptide sequence and programmed into the peptide synthesizer. Fmoc-protected amino acids (5.0 eq) were coupled using HCTU (4.9 eq) as the coupling agent along with NMM (10 eq) to encoded beads (0.32 mmol/g) in DMF in 0.20 mL reaction volume for 8 minutes with shaking set on low. Each amino acid was coupled twice. After each coupling step, terminal Fmoc was deprotected using 40% 4-methylpiperidine (0.10 mL) in DMF for 3 minutes, flushed, and then an additional 20% 4-methylpiperidine (1:1 mixture of 4-methylpiperidine/DMF:DMF, 0.1 mL) was added and reacted for another 5 minutes. The encoded beads were then thoroughly washed with DMF (6 x 0.5 mL). After peptide synthesis, the beads were pooled and the side protecting groups were deprotected using a cocktail consisting of TFA/H_2O/triisopropylsilane (95/2.5/2.5, 0.80 mL) and reacted for 1.25 hours with shaking. The encoded beads were then washed once again with DCM, MeOH, DMF, and then 0.1% PBS TWEEN-20 (0.1% PBST, 0.60 mL). Amino acids were purchased from Millipore. All chemical reagents were purchased from Sigma Aldrich.

2.6 On-bead binding assays

Before the assay, a single aliquot of the pooled encoded beads (0.02 mL from a 0.6 mL stock of suspended beads) was passivated with 2% BSA 0.1% TWEEN-20 PBS pH 7.5 (0.10 mL) overnight in a PCR tube. The beads were pelleted with centrifugation, decanted, and then washed once with 0.1% PBST. Assay buffer (0.1% PBST, final volume = 0.1 mL) was added along with 100 nM of each fluorescently labeled antibody (monoclonal M2-Cy3 anti-FLAG Ab (Sigma), anti-HA Ab Alexa Fluor 647 (Abcam), anti-Myc Ab Alexa Fluor 488 (Abcam)) and then placed on a rotator at ambient temperature for 1 hour. The encoded beads were again pelleted, decanted, and washed with 0.1% PBST 3 times before imaging.

2.7 Bead imaging

Encoded beads were imaged in a custom outfitted Nikon Ti Eclipse microscope as described previously[20,21]. Briefly, bead codes were deconvolved by imaging encoded beads (20 µL) suspended in 0.1% PBST under a Quartz coverslip and glass slide (Fisher Scientific) over 9 emission imaging filters (brightfield-open, 435/40, 473/10, 527/20, 536/40, 546/6, 572/15, 620/14, 630/92, and 650/13 nm, Semrock). A deep UV liquid light guide above the specimen stage provides both deep UV excitation (292 nm, Semrock) for the lanthanide encoding channels and brightfield illumination (409 nm longpass, Semrock) for identifying bead objects within the images. The lanthanide encoding channel emission filters were positioned via a programmable filter wheel (Shutter Instruments) before the emCCD camera (Andor iXon). For peptide binding assays, beads were additionally imaged in fluorescence Cy5, FITC, and Cy3 channels (Chroma) with the deep UV liquid light guide off. Exposure times were optimized for optimal code clustering (Table 1). Images were all taken at 2x binning with a 4x objective.

2.7 Bead image analysis

Bead spectral codes were deconvolved via an imaging pipeline previously developed[20]. Briefly, beads are identified in the brightfield channel via a radius restricted Hough transform. Bead intensities extracted from the bead area are linearly unmixed using solo emitter bead reference standards to determine the most likely linear combination of lanthanides for each observed spectra. Codes are then normalized to an in-bead lanthanide standard (Eu) and are reported as ratios relative to the standard. Subsequently, a cloud point transform is applied to register the reported ratios to the production

target ratios and a Gaussian mixture model is used to fit mean ratios, describe associated covariance and assign each bead to a code. For peptide binding assessment and quantification, a fixed-width annulus circumscribing the identified bead circle (previously from the Hough transform) was used to extract median intensity values in each fluorescence channel per bead. Median intensities were then normalized across the assay to determine relative binding.

3. RESULTS

3.1 High-throughput synthesis of peptide libraries directly on spectrally encoded beads

We have developed a novel technology for synthesizing peptide libraries directly on spectrally encoded beads such that each peptide sequence is uniquely linked to a particular spectral code. This 1:1 association between sequence and codes allows tracking of individual peptides throughout the course of an experiment, thereby enabling high-throughput profiling of many potential protein-peptide interactions within a single small volume (Fig. 1). To begin, we use a microfluidic synthesizer to generate libraries of beads containing multiple distinct ratios of lanthanide nanophosphors, each of which comprises a given spectral code (MRBLEs, or Microspheres with Ratiometric Barcode Lanthanide Encoding). In previous work[20], we have demonstrated the ability to produce and resolve > 1,000 distinct spectral codes with high confidence. Once generated, each code is output to a separate well of a 96 well plate using a custom-built fraction collector integrated within our bead synthesis setup. This plate can be transferred directly into a compatible peptide synthesizer for high-throughput solid-phase Fmoc synthesis of desired peptides on all of the beads within each well, thereby uniquely linking each peptide sequence with a given spectral code (Fig. 1A).

After peptide synthesis, these bead-bound peptide libraries can be pooled and incubated with fluorescently-labeled antibodies of interest for high-throughput measurement of protein-peptide binding interactions (Fig. 1B). After incubation and washing of unbound antibodies, beads are imaged in a custom microscopy setup to optically deconvolve peptide sequences of interest (by "reading out" embedded bead codes) and quantify the amount of bound protein (by directly measuring the intensity of protein fluorescence associated with each bead) without a need for complex downstream processing.

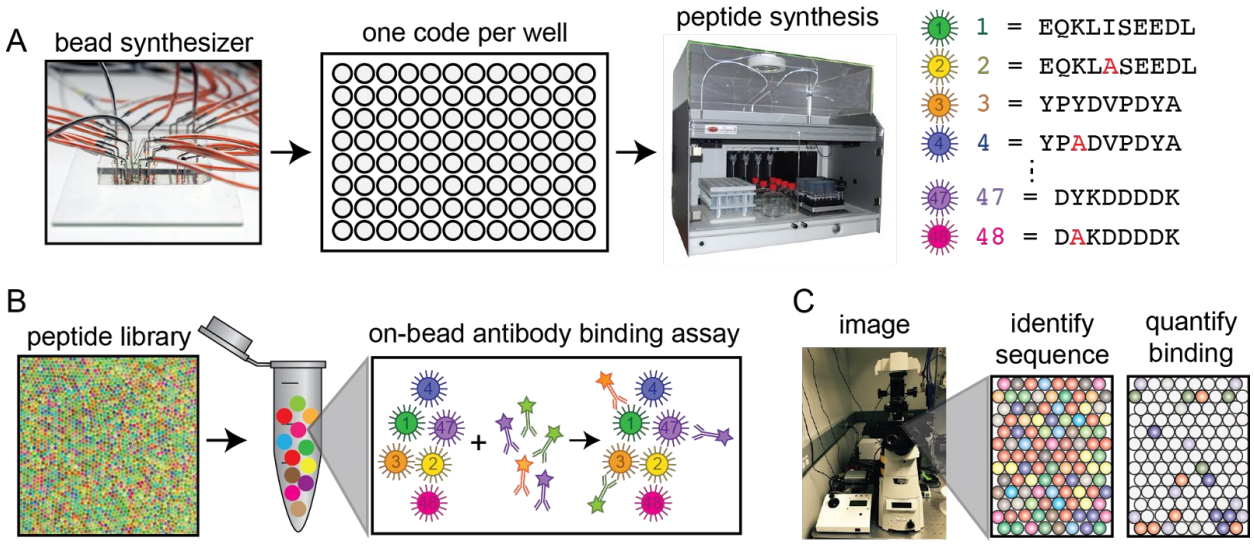

Figure 1. Experimental workflow. **(A)** Spectrally encoded beads (MRBLEs) are produced in a microfluidic bead synthesizer. Each code is output to a separate well of a 96 well plate using a custom built fraction collector. Peptides comprised of myc, FLAG, or HA epitopes and single amino acid substitutions are synthesized on beads with a 1:1 relationship between peptide sequence and spectral code using a high-throughput peptide synthesizer. **(B)** Bead-bound peptide libraries are pooled and incubated with a mixture of anti-myc, anti-FLAG, and anti-HA antibodies labeled with different fluororphores. **(C)** After incubation and washing, beads are imaged to reveal the embedded code (and associated peptide sequence) and the amount of each antibody bound.

3.2 Beads can be functionalized with cysteamine via Michael addition for subsequent solid-phase synthesis

In order to achieve 1:1 peptide functionalization on beads by code, MRBLEs must first be activated via amine functionalization for subsequent peptide synthesis. The encoded beads are synthesized in a custom microfluidic chip from PEG diacrylate pre-polymer and photopolymerized using an initiator (LAP) to form solid hydrogel microspheres. Using the acrylate groups, encoded beads can readily undergo Michael addition[22] with cysteamine to install an amine handle on the beads for solid phase peptide synthesis. In addition to amine functionalization, this same strategy can be utilized to incorporate a broad range of functional handles for bioconjugation, including carboxylic acids and azide or alkyne to list a few. Large biomolecules like proteins, oligonucleotides, antibodies, and lectins can also be anchored to the encoded beads leading to a robust strategy for producing high throughput multiplexed bioassays. We performed cysteamine functionalization reactions sequestered by code with multiple replicates and subsequently assessed amine loading to the bead surface via FMOC quantification[23] via a deprotection strategy.

3.3 Synthesis efficiency can be directly quantified by imaging Fmoc fluorescence

Before proceeding to direct peptide synthesis on beads, amine loading was determined quantitatively via amino acid coupling and FMOC deprotection. Spectrometer results revealed loading for 3 mixed code replicates of 0.262, 0.273, 0.426 mmol/g of encoded beads, with a mean of 0.320 mmol/g that was subsequently used for future synthesis reactions. This loading is well above the pmole range of binding sites per bead, typical of industry standards. Additionally, for qualitative confirmation of FMOC protected amino acid coupling to the bead surface, coupling was observed directly in a microfluidic chamber (Fig. 2B) confirming uniform occupancy of binding sites.

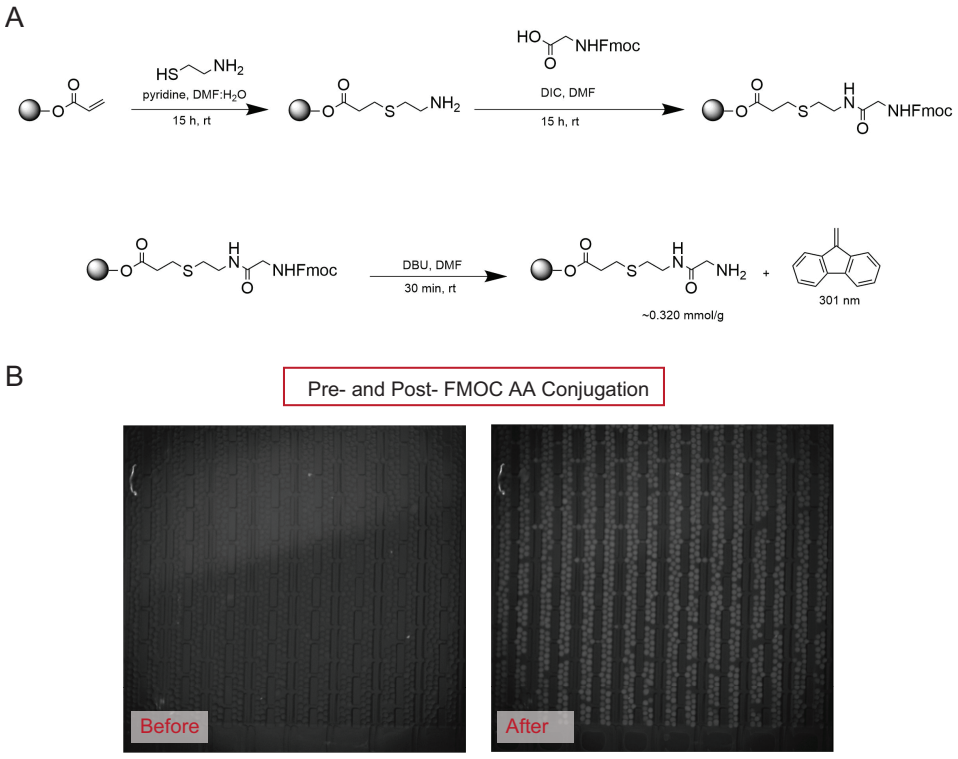

Figure 2. Functionalization schemes. **(A)** Functionalization schematic for cysteamine Michael addition to PEG diacrylate hydrogel encoded MRBLEs (top left), FMOC-protected amino acid addition (top right) and FMOC deprotection (bottom) for fluorescent FMOC quantification in solution as a means to assess bead binding sites. **(B)** Microscopy images of encoded beads before and after FMOC-protected amino acid conjugation (top reaction) post-functionalization in a custom microfluidic device. Beads were imaged under 292 nm excitation and 435 nm emission at the same 2s exposure.

3.4 Embedded codes are robust to chemicals required for peptide synthesis.

Commercially available spectrally encoded beads (*e.g.* Luminex) cannot withstand the harsh chemical conditions required for on-bead synthesis[12,16]. Therefore, we first sought to determine whether the hydrogel MBRLE beads and their embedded spectral codes were robust to on-bead chemical peptide synthesis. To do this, we generated a set of MRBLEs containing 48 codes composed of distinct ratiometric mixtures of Europium (Eu), Dysprosium (Dy), Samarium (Sm), and Thulium (Tm) lanthanide nanophosphors, synthesized a single FLAG peptide epitope (DYKDDDDK) on all beads using standard Fmoc synthesis techniques, and imaged beads before and after synthesis. Both brightfield and lanthanide emission images of beads from before and after peptide synthesis look qualitatively similar (Fig. 3A), suggesting that MRBLEs remain intact throughout Fmoc solid-phase synthesis and subsequent TFA deprotection steps. Bead morphology additionally remains undisrupted before and after synthesis. A more quantitative analysis of measured Dy/Eu, Sm/Eu, and Tm/Eu ratios for this 48-code MRBLE set before and after synthesis confirms that ratiometric levels of lanthanide nanophosphors within the beads remain unchanged, as seen in the code map overlay in Fig. 3B. Taken together, these results establish that MRBLEs can be used as a solid-phase support for direct peptide synthesis, differentiating MRBLEs from other existing spectral encoding platforms.

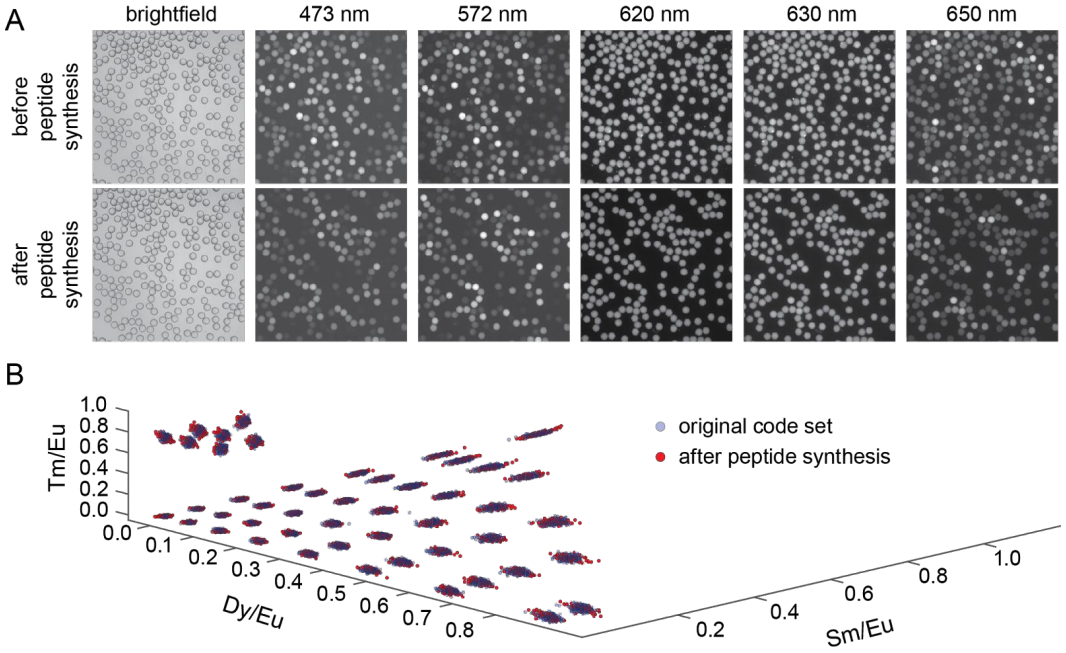

Figure 3. Beads and embedded spectral codes are robust to chemicals required for peptide synthesis. **(A)** Representative images of beads before (top) and after (bottom) on-bead synthesis of a DYKDDDDK (FLAG) peptide. Left image shows brightfield illumination; other images show intensity at lanthanide emission wavelengths under 292 nm deep UV excitation. **(B)** Ratiometric levels of Sm/Eu, Dy/Eu, and Tm/Eu for a 48 code set remain unchanged after FLAG peptide synthesis.

3.5 Bead-bound peptide libraries can be used to profile antibody-peptide binding interactions in direct immunoassays

To demonstrate the utility of these beads for high-throughput measurements of protein-peptide interactions, we sought to detect sequence-specific binding of up to 3 different fluorescently labeled antibodies to a library of spectrally encoded beads containing systematic variations within their cognate epitopes as a proof-of-concept peptide biological multiplexing assay. We generated a library of MRBLEs containing 46 distinct spectral codes and then synthesized both wild-type versions and single amino-acid substitutions of myc, FLAG, and HA peptides on each pool of beads (Table 2). Following peptide synthesis, these bead-bound peptide libraries were pooled and incubated with a mixture of

spectrally distinct fluorescently labeled antibodies (anti-myc-FITC, anti-HA-Cy5, and anti-FLAG-Cy3). After washing beads to remove unbound antibodies, bead-bound peptide libraries were imaged to identify the embedded bead codes (and thus, the peptide sequence associated with each bead) and quantify the amount of each antibody bound (Fig. 4). Qualitatively, we could see that each bead was recognized by only a single type of fluorescently labeled antibody, establishing that binding of fluorescently-labeled antibodies is specific to particular peptide sequences and suggesting that there is limited cross-reactivity between these three antibodies (Fig. 5A). Quantitatively, these measurements revealed that each antibody bound with high specificity and minimal cross-reactively to its cognate epitope and variants, with very little observed background binding (Fig. 5B). Importantly, each pooled library measurement required only 100 nM of each antibody per 100 uL assay containing hundreds of beads, establishing that this multiplexed peptide bead-based assay can return valuable information about binding interactions using extremely small amounts of material (100 nM of each antibody within a 100 µL total volume).

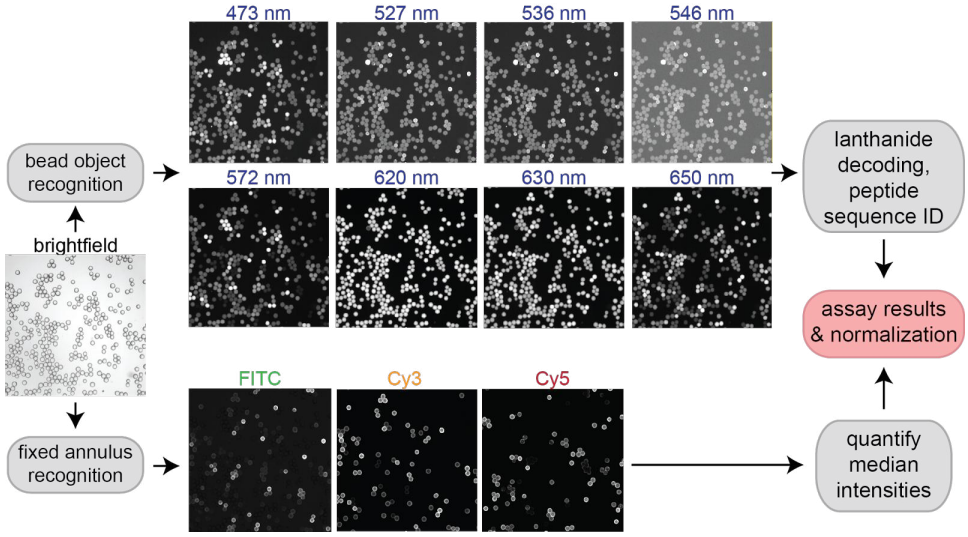

Figure 4. Imaging workflow. Schematic of image analysis scheme used to assay antibody/peptide binding. Bead objects are recognized, lanthanide codes are called and matched to known peptide sequences and median counts for antibody bindings are mapped to deconvolved bead codes for final assay quantification. Representative full-stack images are shown of a 46 MRBLE code set over brightfield, 8 emission filters for lanthanide code calling, and 3 fluorescence filters for antibody binding quantification in a peptide multiplexing assay. Images are shown post-incubation with a mixture of labelled anti-myc, anti-FLAG, and anti-HA antibodies.

4. DISCUSSION

Here, we have demonstrated the ability to directly synthesize a library of specific peptides on spectrally encoded beads, laying the foundation for a wide variety of future high-throughput multiplexed protein/peptide interaction experiments using very small amounts of material. In future work, we seek to improve our confidence in binding measurements in several ways. A significant advantage of bead-based assays over spatial arrays is the fact that peptides can be cleaved from solid supports and characterized via mass spectrometry to assess peptide quality. We plan to incorporate a quality control step to assess peptide synthesis, thereby making it possible to distinguish whether a loss of signal results from an amino acid substitution that truly ablates binding, or from technical issues with synthesis (*e.g.* the creation of truncated products). In addition, we plan to incorporate normalization schemes to correct for sequence-specific variations in peptide synthesis efficiency and allow a semi-quantitative estimate of interaction affinities based on measured intensities. While we observe low error associated with each antibody affinity measurement shown here, we have observed significant inter-assay variability from possible day-to-day differences in synthesis conditions or variation in bead batch production. Addressing inter-assay variability could be achieved by tightening bead size distributions and using more precise fluid manipulators for high-precision bead functionalization. We have begun efforts to lower bead size production variability using fluorinated oils, and plan on further standardizing bead functionalization with bulk fluid handlers and plate based functionalization schemes.

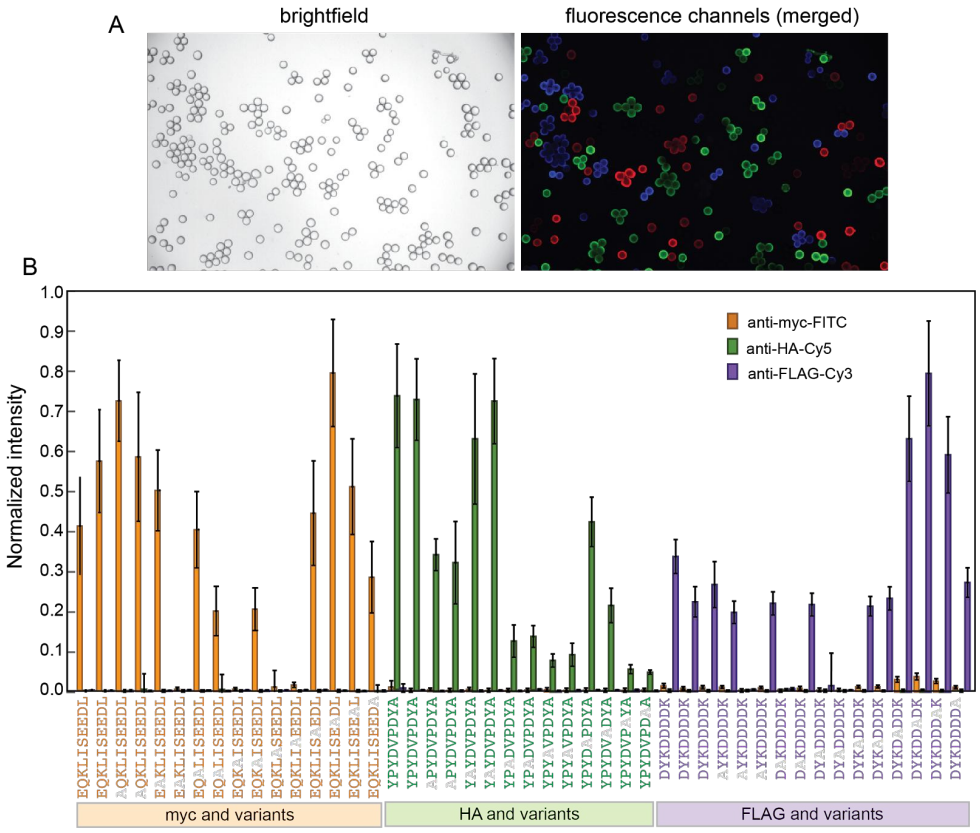

Figure 5. Bead-based assay demonstrating highly multiplexed measurements of protein binding specificities. **(A)** Brightfield (left) and merged composite image (right) showing binding of anti-myc-FITC, anti-HA-Cy5, and anti-FLAG-Cy3 to beads containing either myc, HA, or FLAG peptides. **(B)** Median normalized anti-myc-FITC (orange), anti-HA-Cy5 (green), and anti-FLAG-Cy3 (purple) intensities for all beads containing specific peptide epitopes (myc, HA, and FLAG) and single-amino acid substitutions.

In addition to improving the technical aspects of our technology, we also hope to broaden the scope of its applicability by embarking on more challenging biological assays, including measurements of weak and transient protein-peptide interactions thought to be most important for cellular signaling pathways. The chemical synthesis method described here holds particular promise for understanding cellular signaling, as it enables direct probing of how post-translationally modified amino acids incorporated at particular sites affect the strength and stability of binding interactions.

5. CONCLUSION

The results presented here establish the feasibility of using spectrally encoded beads as a novel support for solid-phase peptide synthesis. This work extends the potential of one-bead, one-compound libraries by enabling high-throughput analysis of protein-peptide interactions without a need for downstream recovery and molecular decoding, as beads of interest can be simply imaged to determine the identity of their associated peptides.

Table 1: Exposure times for lanthanide and fluorescent imaging channels for MRBLE assays.

Imaging Channel	**Exposure time (ms)**
Brightfield	10
Ex 292 - Em 435	1000
Ex 292 - Em 473	2000
Ex 292 - Em 527	1000
Ex 292 - Em 536	1000
Ex 292 - Em 546	1000
Ex 292 - Em 572	750
Ex 292 - Em 620	650
Ex 292 - Em 630	150
Ex 292 - Em 650	550
Cy3	100
Cy5	400
FITC	300

Table 2: Code-to-peptide mapping assignment for the 46 code multiplexed peptide/antibody MRBLE assay

Bead Code	Peptide	Bead Code (cont.)	Peptide (cont.)
1	EQKLIAEEDL	24	YAYDVPDYA
2	EQKLISAEDL	25	YPADVPDYA
3	EQKLISEADL	26	YPYAVPDYA
4	EQKLISEEAL	27	YPYDAPDYA
5	AQKLISEEDL	28	YPYDVADYA
6	EAKLISEEDL	29	YPYDVPDYA
7	EQALISEEDL	30	YPYDVPDYA
8	EQKAISEEDL	31	DYADDDDK
9	EQKLASEEDL	32	DYKADDDK
10	EQKLISEEDL	33	DYADDDDK
11	EQKLISEEDA	34	AYKDDDDK
12	AQKLISEEDL	35	DYKADDDK
13	EAKLISEEDL	36	DYKDADDK
14	EQALISEEDL	37	DYKDDADK
15	EQKAISEEDL	38	DYKDDDAK
16	EQKLISEEDL	39	AYKDDDDK
17	APYDVPDYA	40	DAKDDDDK
18	YAYDVPDYA	41	AYKDDDDK
19	YPADVPDYA	42	DAKDDDDK
20	YPYAVPDYA	43	DYKDDDDK
21	YPYDVPAYA	44	DYKDDDDA
22	YPYDVPDAA	45	DYKDDDDK
23	APYDVPDYA	46	DYKDDDDK

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