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Article

Single Bead Labeling Method for Combining Confocal Fluorescence On-Bead Screening and Solution Validation of Tagged One-Bead One-Compound Libraries

Martin Hintersteiner,[1,2] Thierry Kimmerlin,[2] Frank Kalthoff,[2] Markus Stoeckli,[3] Geraldine Garavel,[2] Jan-Marcus Seifert,[2] Nicole-Claudia Meisner,[2,3] Volker Uhl,[2] Christof Buehler,[2,5] Thomas Weidemann,[2,4] and Manfred Auer[1,2,*]
[1]University of Edinburgh, School of Biological Sciences, The King's Buildings, CH Waddington Building 3.07, Mayfield Road, Edinburgh EH9 3JR, UK
[2]Innovative Screening Technologies unit, Novartis Institutes for BioMedical Research, Brunnerstrasse 59, A-1235 Vienna, Austria
[3]Novartis Institutes for BioMedical Research, Novartis Campus Forum 1, 4056 Basel, Switzerland
[4]Institute of Biophysics, Biotechnologisches Zentrum der TU Dresden, Tatzberg 47-51, D-01307 Dresden, Germany
[5]Supercomputing Systems AG, Technoparkstrasse 1, CH-8005 Zurich, Switzerland
*Correspondence: manfred.auer@ed.ac.uk
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SUMMARY

Screening of one-bead one-compound libraries by incubating beads with fluorescently labeled target protein requires isolation and structure elucidation of a large number of primary hit beads. However, the potency of the identified ligands is only revealed after time consuming and expensive larger scale resynthesis and testing in solution. Often, many of the resynthesized compounds turn out to be weak target binders in solution due to large differences between surface and solution binding affinities. For an industry style high-throughput screening (HTS) process a high false positive rate is detrimental. We have therefore combined single bead and single molecule/single cell techniques into an integrated HTS process in which the picomole amount of substance contained on one isolated hit bead is sufficient for quality control, structure determination, and precise affinity determination to the target protein in solution.

INTRODUCTION

Small molecular ligands are important tools for studying complex biological functions (Chen et al., 2006; Xu et al., 2008). The identification of small molecular ligands for any biochemically produced protein has been assigned as one of the most significant challenges in the field of chemical biology (Schreiber, 2005). Despite all recent technological advances in high-throughput screening and combinatorial synthesis, the question of how to probe chemistry versus biology most efficiently still remains a moving target. The need for quantitative biophysical characterization adds an additional level of complexity to any screening process. Therefore, research efforts both in academia and industry were focused on the development of novel screening concepts (Diaz-Mochon et al., 2006; Blackwell et al., 2001; Bradner et al., 2006; Clemons et al., 2001; Duffner et al., 2007; Meisner et al., 2004; Metzger et al., 2006; Muckenschnabel et al., 2004; Sedlacek and Chen, 2005; Urbina et al., 2006; Vegas et al., 2008; Winssinger et al., 2008; Zehender et al., 2004). However, none of these concepts are fully integrated, miniaturized, and quantitative.

One-bead one-compound (OBOC) libraries produced by combinatorial synthesis are still the most efficient and most flexible method to generate a large number of substances. If the molecules produced by OBOC chemistry could be screened against targets directly at the site of synthesis and if this primary screening method could be connected to hit isolation, structure determination, quality control, and affinity determination in solution, the above mentioned three main criteria would be fulfilled. Furthermore, this "single bead process" would only rely on ~50 ng of each individual library compound. Resynthesis efforts would be focused on the most potent compounds. Such a screening process would dramatically gain if it was directly linked to validation in cells and model organisms.

The basic idea of screening ligands directly on bead appeared in the literature as early as 1991 (Lam et al., 1991). Many steps have since then been taken (Kodadek and Bachhawat-Sikder, 2006; Lathrop et al., 2007; Lehman et al., 2006; Lim et al., 2007; Meisner et al., 2009; Meldal, 2002; Paulick et al., 2006; Pei and Wavreille, 2007; Song et al., 2003; Sweeney et al., 2006; Uhl et al., 2002; Youngquist et al., 1994; Zhang et al., 2008) to realize this concept. Reported progress ranges from optimized screening conditions to library decoding strategies and the use of entire cells for on-bead screening (Peng et al., 2006; Udugamasooriya et al., 2008; Wang et al., 2005a, 2005b; Chen et al., 2009). However, to develop bead based screening into a process that combines both industry requirements of cost effectiveness and throughput and the highest standards in quantitative biology, the following needs had to be addressed. First, an automated detection method for protein-ligand interactions on bead had to be established. The key parameters to address were quantification of bound fluorescent protein at the

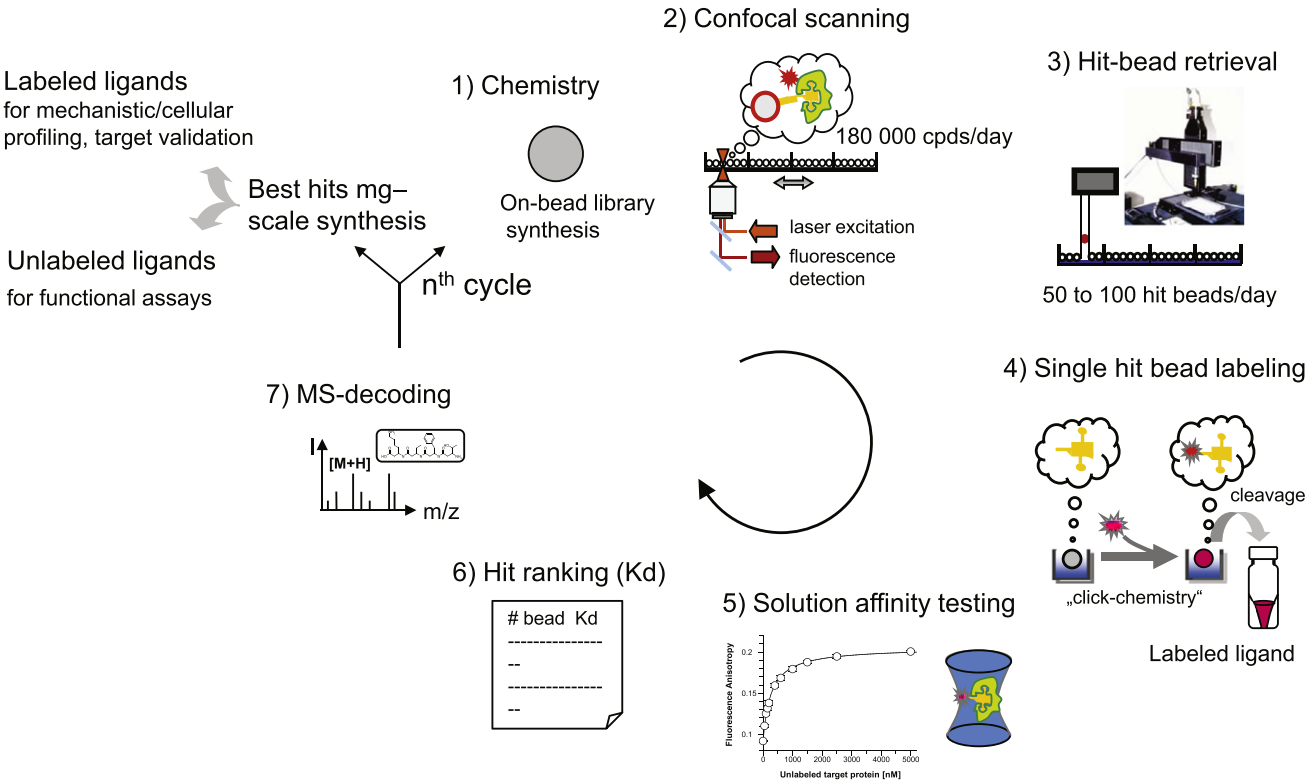

Figure 1. The ICB Discovery Cycle Comprises Seven Steps and Combines On-Bead and Solution Screening

Step 1: design and synthesis of OBOC libraries by combinatorial chemistry. Step 2: hit bead detection by CONA. Step 3: semiautomated hit beads isolation and deposition (one bead per vial). Step 4: conversion of bead bound compounds into fluorescent ligands by site-specific labeling and compound cleavage. Step 5: affinity measurement in homogenous solution by titration of labeled hit compounds with unlabeled target protein using a miniaturized confocal fluorescence fluctuation assay. Step 6: hit bead ranking. Step 7: MS-based structure determination.

bead surface and reliable distinction between hit beads and autofluorescent beads. Second, a direct link between on-bead binding and affinity determination in solution needed to be developed. Third, the chemical method for combining surface screening and solution testing needed to be flexible enough to include the option for profiling identified ligands in cellular assays and in validation assays further downstream.

Herein we describe a new screening methodology, "integrated chemical biophysics" (ICB), which integrates library design and synthesis, automated confocal nanoscanning (CONA) for preselection of hit compounds on the solid surface as primary screen, quantitative testing of single hit bead-derived compounds in solution, as well as structure determination and quality control (Figure 1). This is achieved by converting all compounds on picked hit beads into fluorescent ligands. With a free choice of color, additional chemical tagging possibilities, and an affinity (Kd) connected to each compound, cellular validation by high resolution confocal imaging, microspectroscopy, or functional assays is immediately possible. The ICB process is demonstrated by screening a >100,000 member library of designed α,β-phosphopeptides against the Grb2 SH2 domain. The identification of ligands with nano- to micromolar affinity (Kd) for Grb2 SH2 in solution from this library without prior resynthesis shows the utility and flexibility of the ICB method and its unique power in producing a reliable and strictly quantitative high-throughput SAR in a primary screening campaign.

RESULTS

Automated CONA and Bead Picking

Current on-bead screens are performed on standard (fluorescence) microscopes or on the COPAS bead sorter (Union Biometrica).

These methods detect integrated fluorescence intensities, from the entire volume of individual (TentaGel) beads. Hit bead identification is hampered by the varying autofluorescence levels occurring in combinatorial OBOC libraries (Ding et al., 2006; Olivos et al., 2003). (Although this problem is less pronounced with PEG-based resins, the increased mechanical stability of TentaGel beads is highly advantageous during library synthesis and on-bead screening.) While autofluorescence is distributed throughout the bead matrix, the limited pore size of TentaGel beads largely prevents the fluorescently labeled target proteins (>10 kDa) from diffusing inside the bead in the time frame of incubation and screening (<24 hr). Therefore, to limit detection of fluorescence artifacts and to allow precise quantification of target binding to compounds presented at the bead surface the on-bead screening relevant binding signal for TentaGel beads must be recorded within the outer few micrometers of

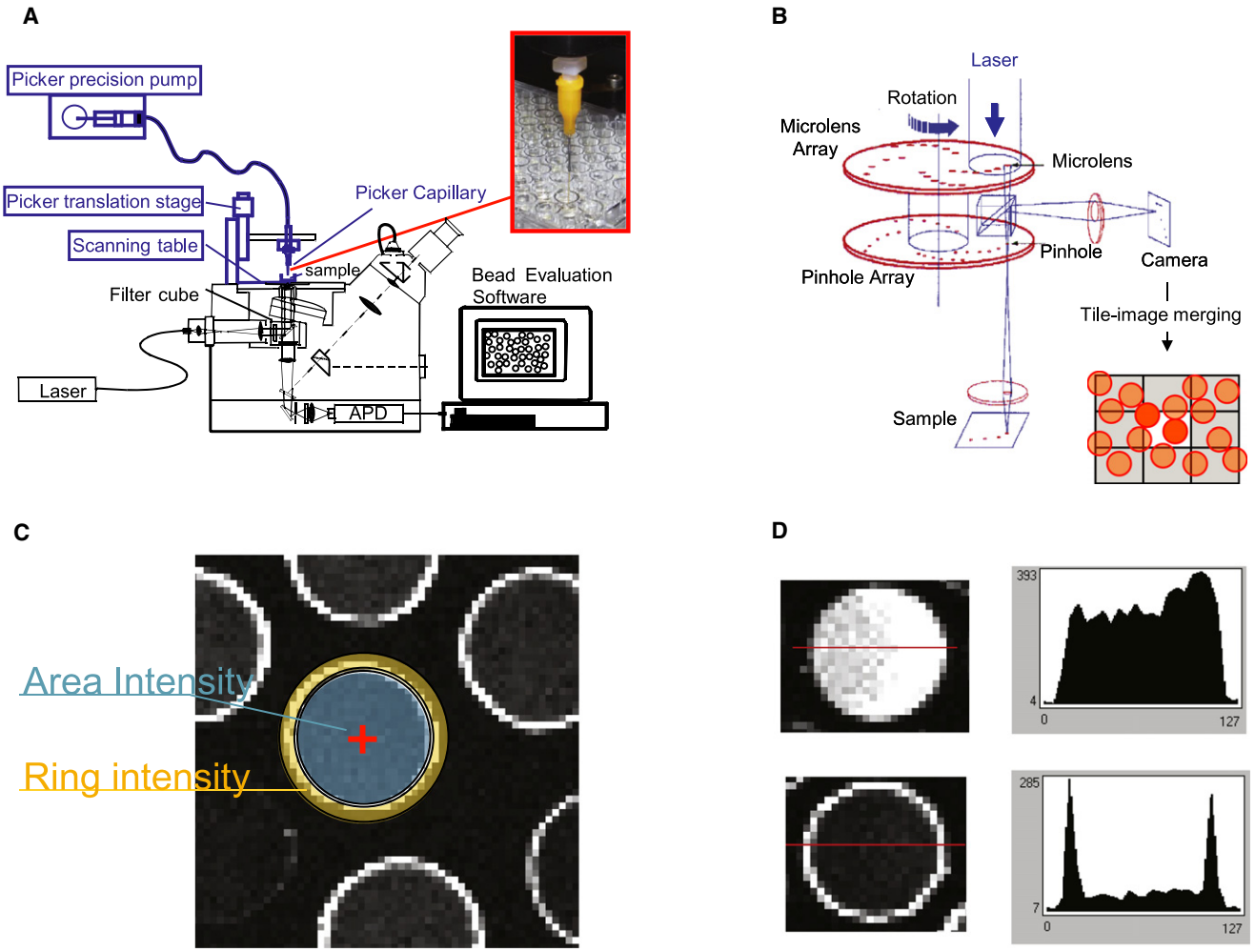

Figure 2. On-Bead Screening by CONA

(A) Schematic layout of the PickoScreen (PS0x) instruments. The PickoScreen instruments are confocal microscopes equipped with a scanning table and a picking robot. The picking robotics operates a capillary (depicted in the inset), through which individual hit beads are sucked up from the bottom of the microtiter plate via pneumatic vacuum.

(B) In an advanced high-speed version of the PickoScreen design (PS04), the traditional confocal optics was replaced by a Nipkow spinning disc, which uses multiple pinholes for parallel sampling of individual picture frames. The tile pictures are finally merged to generate whole-well images.

(C) During CONA the stage is moved and the confocal focus is held just below the equatorial plane of beads. The beads appear as round objects in the scan image. For each bead two parameters are registered: the fluorescence intensity in the bead interior (area intensity) and the intensity in a small ring on the outer edge of the beads (ring intensity).

(D) The 2–5 μm scanning resolution in CONA allows to efficiently distinguish between beads with a high level of autofluorescence (top) and hit beads with bound target protein on the outside (bottom).

a bead. We reasoned that by measuring spatial intensity distributions along the equatorial plane of a bead with confocal imaging, much higher signal to noise ratios can be achieved, as compared to standard integrative fluorescence methods.

Together with Evotec Technologies (formerly ET, now Perkin Elmer) we have developed three fluorescence microscopes based on the Insight Reader platform for single molecule spectroscopy and dedicated them to three essential steps in bead based screening: PS02, an instrument for high resolution confocal scanning and bead picking; PS04, an instrument for high speed CCD-based confocal bead imaging; and PS03, an instrument for high resolution microspectroscopy. (Figure 2A). The technical aspects of the PS02 and PS01 instruments are described in detail in Hintersteiner et al. (2009). Briefly, each of the ''PickoScreen'' instruments consists of a modified Olympus IX70 microscope with an integrated confocal multicolor detection system. The PS02 instrument is equipped with a high precision motorized scanning table holding an integrated sample compartment. Other than in conventional confocal laser scanning microscopes the laser beam for fluorescence excitation remains fixed while the sample compartment is moved during image acquisition. This setup offers the advantage that a larger sample area can be imaged without optical distortion. In an advanced modification, fast multiparallel confocal imaging has been realized in PS04 by using a Nipkow spinning disc with multiple pinholes and CCD cameras for signal detection (Figure 2B).

For primary on-bead screening the wells of a 96 well microtiter plate are filled with ~1 mg of TentaGel beads (90 μm diameter and ~100 picomoles loading per bead), equaling ~2000 beads per well. The resulting monolayer of beads is then incubated with ~1–50 nM fluorescently labeled target protein for several hours and automatically scanned well by well. Thus, the scanning of a 96 well microtiter plate (~180,000 beads) takes less than 7 hr on the fast Nipkow-based PS04. By moving the 1–2 × 0.4 μm sized confocal focus through the equatorial plane of the beads, spatially resolved fluorescence intensities are collected and beads are represented as disc-like objects (Figure 2C). A proprietary pattern recognition software (BeadEval; Perkin Elmer) allows analysis of the primary screening image by locating each individual bead's position (x/y coordinates) and by resolving two characteristic parameters: the average fluorescence intensity of the bead disc's interior (area intensity) and the mean intensity on the bead surface at the height of the slicing confocal volume (ring intensity, usually 5 μm). Hit beads, i.e., beads with surface-bound target protein, exhibit increased fluorescence intensity on the outer surface of the bead, compared to the average fluorescence intensity in the interior. Because of optical distortions caused by the bead matrix changed refractive index, bead ring and interior intensity cannot be quantitatively compared. However, real hit beads appear as ringed discs in the image, as compared to autofluorescent beads, which appear entirely bright (Figure 2D). Most importantly, the ring intensity determination allows the ranking of all beads in each well based on the amount of fluorescent target protein bound to the immobilized compounds. The problem of reliable single hit bead retrieval from a large population was solved via bead picking by a robot-operated glass fiber capillary. For semiautomated retrieval of hit beads a capillary is precisely positioned at the xy coordinates of the bead via a robot arm and the bead is sucked into the capillary by hydraulic vacuum. The hit bead is then dispensed into a vial. This picking procedure takes on average 1 min per bead.

Single Bead Derivatization by Post-Synthesis-Post-Screening Labeling

Although primary on-bead screening with CONA allows for quantification of target-ligand complex formation on individual hit beads, this parameter can only be used for "preliminary" ranking of hit compounds due to the following reasons. First, the compound density on bead is high (mM). Ligands are present in vast excess over the target protein. Therefore, also low affinity ligands will be identified. This, per se, essential advantage of the on-bead screening process makes it difficult to define a low affinity cut-off for bead selection. Second, there are a number of parameters influencing the molecular recognition between protein and bead immobilized ligands: altered relative entropy and enthalpy contributions on a compound by compound basis may lead to an increased affinity when the compound is immobilized on the bead. Other factors include molecular tumbling, pH of the microenvironment, or protein unfolding in the bead matrix. Third, the protein-ligand complex formation and hence the observed fluorescence signal will not only depend on affinity but will also be influenced by factors not related to molecular recognition. Such factors include compound purity, aggregation, or ion exchange effects. Depending on the reaction efficiency during compound synthesis the final number of compounds on individual beads may vary substantially. Therefore, a process with all primary hit compounds resynthesized before they can be tested in solution will include low affinity binders and false positives. Furthermore, the other strategy chosen in the current on-bead screening literature, i.e., decoding a large number of hit compounds and synthesizing only consensus motifs, does not guarantee that the highest affinity binder is identified and does not allow a systematic structure-activity relationship analysis. To solve this process critical "resynthesis issue" a reliable and stable method for measuring the affinity between the target and single bead-derived compound is needed. The best possible way to achieve this goal is via incorporation of a highly sensitive label into each of the library compounds. We therefore introduced a post-synthesis-post-screening labeling (PS/PS labeling) step into our chemical OBOC strategy (Figure 3A). Thereby, labeled target protein and unlabelled library compounds are used for primary screening on bead followed by a switch to testing unlabelled target for binding to labeled hit compounds in solution. The 1,3-dipolar cycloaddition of terminal alkynes and azides to form 1,4-triazoles, known as "click reaction" (Kolb and Sharpless, 2003; Tornoe et al., 2002; Tornoe and Meldal, 2001) seemed particularly suitable for such a single bead labeling step, due to its high efficiency and the orthogonality of terminal alkynes to many other functional groups. A spacer was incorporated to achieve a separation between the reporter group and the binding face of the compound. For testing the feasibility of this concept, a batch of 100 beads containing a test peptide as well as randomly picked individual beads from an on-bead library were subjected to the PS/PS-labeling procedure (see Figures S2A, S2B, and S3 available online). Using the ascorbic acid/copper sulfate reagent combination (Rostovtsev et al., 2002) and an azide-functionalized tetramethyl-rhodamine (TMR) **1** as dye (for compound synthesis, see Supplemental Experimental Procedures and Figure S1), batch labeling of ~100 beads and derivatization of individual beads was achieved with near quantitative yields and excellent reproducibility. On average, 54 ± 23 picomoles of PS/PS-labeled compound were obtained from one 90 μm TentaGel bead (Table S1). Because the labeling site is introduced in the first step during compound synthesis, the generic PS/PS-labeling method also provides a valuable tool for analyzing the purity and homogeneity of OBOC libraries by HPLC.

Combining On-Bead Screening and Solution Confirmation

Having established all key elements, most importantly, CONA and single bead PS/PS labeling, we integrated them into a ligand identification and validation process (ICB). ICB comprises seven steps and yields hit structures along with their quantitative affinity information (Kd) to the target protein in solution (Figure 1). The process starts with the design and synthesis of OBOC libraries on 90 μm TentaGel beads by combinatorial chemistry. For the on-bead screening step, each well of a 96 well microtiter plate is filled with 1 mg of resin beads (~2000 beads per well). The further process steps are CONA, hit bead retrieval, single bead labeling, confocal fluctuation analysis methods for solution testing, and Kd determination, quality control, and structure

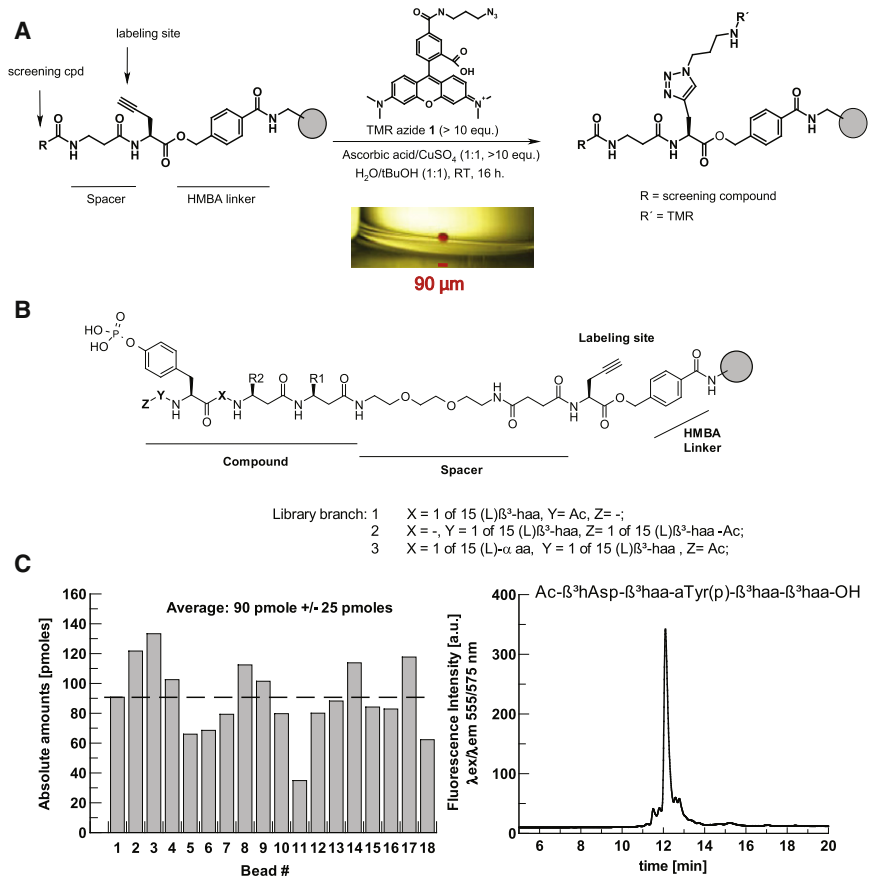

Figure 3. PS/PS Labeling and Phosphopeptide Library Design

(A) PS/PS labeling is used to achieve a direct link between on-bead screening and the quantitative affinity determination of single bead-derived ligands for their target protein in solution. The chemical setup contains a labeling site, separated by a spacer unit from the screening compounds. After on-bead screening with CONA and bead picking, individual hit beads are treated with an azide modified dye, e.g., TMR-N_3 **1**, to convert all compounds into fluorescent ligands for further affinity testing via fluorescence spectroscopy.

(B) Design of phosphopeptide library *pYL1*: a library of mixed α/β-phosphopeptides was generated on HMBA TentaGel beads. The library contained two branches with four combinatorial positions and one branch with three combinatorial positions around the central α-phosphotyrosine.

(C) Quality control of the phosphopeptide library *pYL1*: 18 beads were randomly picked from library *pYL1* and subjected to PS/PS labeling (left). Determined by HPLC, on average, 90 ± 25 picomoles of fluorescently labeled peptide were obtained from a single bead. The average purity of library compounds was >70%; an exemplary chromatogram is shown on the right.

determination by MS methods. After primary CONA screening and PS/PS validation from single beads, the best scoring hits can be resynthesized in milligram quantities with or without the spacer and fluorophore tagging site. This opens up multiple ways for testing the newly identified ligands in functional, (single cell) imaging, or model organism secondary assays. To demonstrate the power of this process, we designed and synthesized an SH2 domain-tailored library of mixed α/β-phosphopeptides and screened it against SH2 domain targets, such as Grb2.

Library Synthesis

SH2 domains constitute an essential class of cell signaling interaction domains. They recognize short peptide stretches with only a few residues C- and N-terminal to the phosphotyrosine (Table S2). Limited cell permeability and plasma stability are the main limitations for using α peptides for pharmaceutical applications. In contrast, β peptides are highly stable toward enzymatic degradation (Wiegand et al., 2002). We have therefore designed and synthesized a OBOC library of more than 100,000 mixed α/β-phosphopeptides on the PS/PS-labeling platform, described above. The library design contained three distinct branches, each intended to mimic different regions of the consensus motif (Figure 3B and Figures S4 and S5). Two library branches consisted of pentapeptides entailing four combinatorial sites plus the phosphotyrosine and the third branch consisted of tetrapeptides entailing three combinatorial sites plus phosphotyrosine. The library was synthesized by the split and mix procedure, using 15 amino acids with proteinogenic side chains in each position (the set of 20 natural amino acid side chains excluding Leu, Cys, Met, His, and Gly). After the last coupling step, the sublibraries were kept separated. Therefore, the entire library comprised 45 sublibraries (three times 15 building blocks; Table S3). For an initial quality control of the library, 18 randomly picked beads were subjected to the single bead derivatization protocol, as described above. According to HPLC analysis after cleavage from resin, these samples contained 90 ± 25 picomoles of fluorescent compound with an average main peak purity of >70% (Figure 3C).

The ICB Screening Process with Grb2 as Target

For CONA on-bead screening of the entire phosphopeptide library, the 45 sublibraries were distributed into the wells of a 96 well microtiter plate (1 mg of resin, i.e., ~2000 beads per well). Two wells were used per sublibrary for the two larger pentapeptide branches and one well per sublibrary for the third tetrapeptide branch (for well distribution see Figure S6 and Table S4). Thus, the entire screening plate consisted of 75 wells with ~150,000 beads (i.e., individual compounds). Following statistical considerations, the screening plate covered between 70% and 100% of the total diversity of the library, which is 104,625 phosphopeptides (for details, see Supplemental Experimental Procedures and Figures S7 and S8). (It is an often encountered misconception in the context of library synthesis and screening that only theoretical diversities are described for a library, without taking into account the number of beads used during chemical synthesis and screening.) After a short preblocking step with a gelatine-based blocking buffer, the beads were incubated with Cy5-labeled Grb2-SH2 (5 nM protein) at 4°C

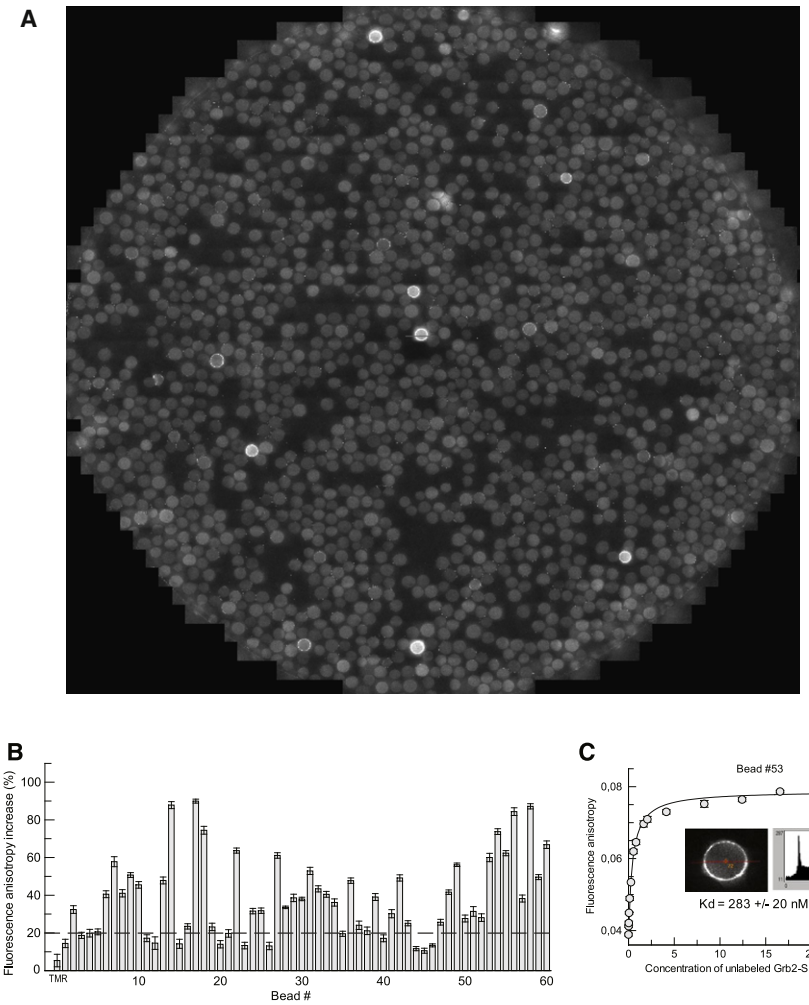

Figure 4. Identification of Grb2 Ligands from the Phosphopeptide Library pYL1

(A) The sublibraries of the phosphopeptide library pYL1 were screened with Cy5-labeled Grb2 by CONA. Hit beads in each well were counted, ranked according to their fluorescence ring intensity, and retrieved by CONA bead picking (an exemplary scanning image of a well is shown).

(B) After PS/PS labeling, all hits were analyzed for binding to unlabelled Grb2-SH2 (at 24 μM) in a 2D-FIDA anisotropy assay. The anisotropy increase upon Grb2-SH2 addition (in percent, relative to a sample without Grb2-SH2) is shown. TMR-N_3 was used as a control for viscosity-induced anisotropy increase. A fluorescence anisotropy increase of 20% (three times the increase observed with TMR-N_3; dotted line) was used as the threshold for hit selection. Data points represent mean percent fluorescence anisotropy increases, averaged over ten individual measurements ± SD.

(C) Titration curves with Grb2-SH2 were recorded for the samples with the highest fluorescence anisotropy increase in the single point measurements. The titration curve for the highest affinity ligand along with the original bead scan image and intensity profile (bead #53, inset) is depicted. Data points represent mean fluorescence anisotropy, averaged over ten individual measurements ± SD.

overnight. The on-bead screen was run on the PS04 instrument in less than 6 hr of scanning time. The automated analysis of ring and area intensities for all beads in each well revealed a total of 238 hit beads (hit rate 0.16%) with a "ring intensity" that exceeded the background signal at least 5-fold. Counting the number of hit beads per well allowed the prioritization of 16 wells, as the predominantly responding sublibraries, for bead picking. Figure 4A shows one exemplary well with several hit beads. From the selected wells the 60 beads with the highest ring intensities were picked, using the capillary based bead-picking procedure of the PS04 instrument.

Solution Confirmation of Primary Hit Beads by Confocal Fluorescence Fluctuation Analysis

Various confocal fluorescence methods are suitable for the solution assay after single bead cleavage of fluorescent derivatives of hit compounds. Overall, the fastest and most reliable method is 2D-FIDA anisotropy (Kask et al., 2000). Therefore, single bead-derivatized TMR-labeled hit compounds, in amounts between 27 and 108 picomoles, were dissolved in 20 μl of water and 20% acetonitrile. By taking 1 μl only of each sample and further 1:500 dilution, suitable concentrations for the 2D-FIDA-r assay was reached. Single-point measurements for Grb2-SH2 binding in a low-volume 384 well microtiter plate (7 μl assay volume and 24 μM Grb2-SH2 concentration) led to anisotropy increases of 5% to 90% (Figure 4B). Derived from the 60 picked hit beads, 46 samples showed reasonable anisotropy increases upon protein addition. We selected the 12 samples with the highest anisotropy increases for recording complete titration curves. The 2D-FIDA anisotropy titrations of these samples resulted in Kd values between 230 nM and 14 μM for 11 compounds (Figures S9 and S10). One sample was excluded from further follow up due to compound aggregation. The single bead-derived titration curve for the highest affinity binder (bead 53) together with the confocal scan image of the original hit bead is depicted in Figure 4C.

MALDI Decoding, Resynthesis, and Reconfirmation of Identified Hits

MALDI-MS-based decoding of the ten hit compounds with dissociation constants below 5 μM resulted in proposed hit structures for eight compounds (Figure 5A and Table S5). The peptide sequence for two hits was not decodable. A total of 19 candidate peptides, GhT 1–19, was resynthesized on 50 mg resin, and the compounds were retested for binding to Grb2 on bead and in solution (Figures 5B and 5C, Tables S6 and S7, and Figure S11). The MS-based structure decodes and single bead-derived affinities (Kds) of the resynthesized compounds were verified with the exception of one compound. Most importantly, the quantitative binding data revealed from primary ICB screening and from the confirmation cycle were in excellent

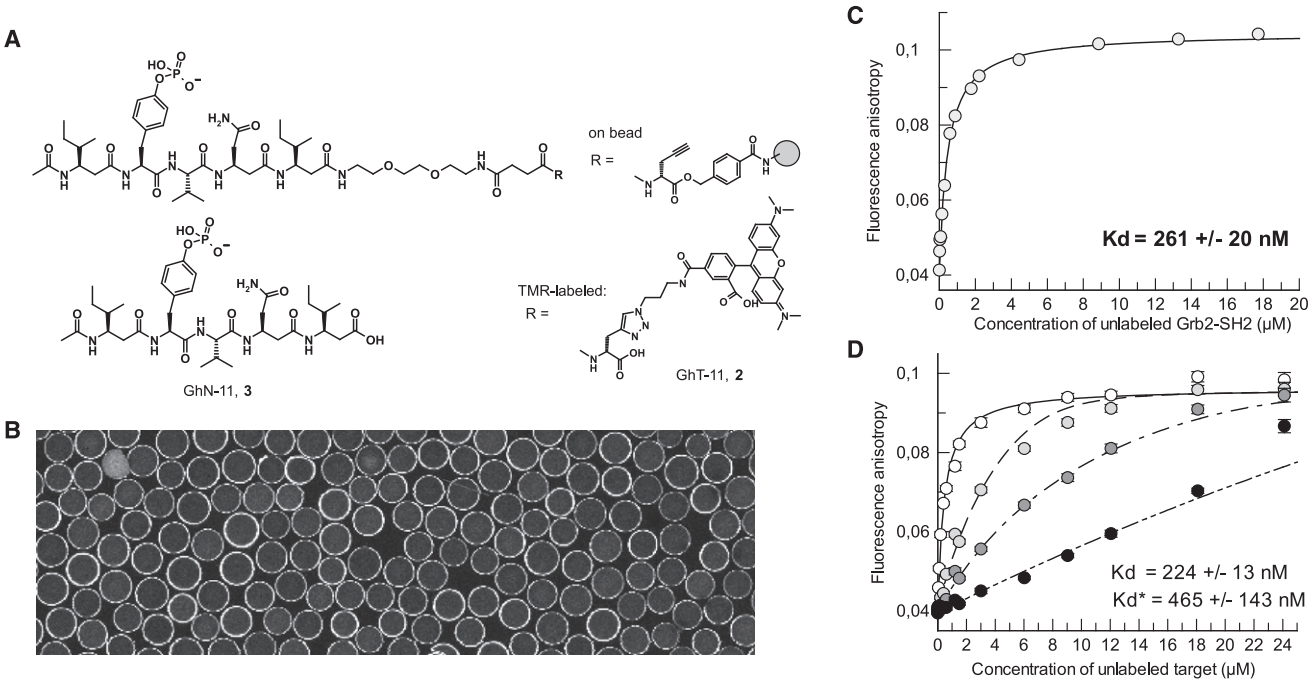

Figure 5. Hit Bead Decoding, Resynthesis, and Retesting On Bead and in Solution

(A) MALDI-MS based structure determination and decoding revealed GhT11 **2** as the ligand structure linked to the hit bead #53.

(B) GhT11, resynthesized in a larger batch on TentaGel beads, gave rise to homogenous ring formation in the on-bead assay. A small section of a CONA image selected from a well, filled with GhT11 **2** containing beads, and incubated with Cy5-labeled Grb2-SH2 is shown.

(C) When labeled with TMR-N_3 using the click reaction, the resynthesized ligand GhT11 **2** bound to Grb2-SH2 with an affinity (Kd) of 261 ± 20 nM, confirming the original affinity determination with the single bead-derived material. Data points represent mean fluorescence anisotropy, averaged over ten individual measurements ± SD.

(D) Competition titration for binding of the unlabelled Grb2 ligand GhN-11 **3** to Grb2-SH2 with GhT-11 **1** as surrogate ligand. The unlabelled ligand GhN-11 **3** exhibited an affinity (Kd) of 465 ± 143 nM for Grb2-SH2. Data points represent mean fluorescence anisotropy, averaged over ten individual measurements ± SD.

agreement. To complete the discovery cycle, outlined above, the best compound (GhT-11, **2**) was also synthesized in unlabelled form (GhN-11, **3**, without labeling site and spacer). A 2D-FIDAr titration of a mix of the unlabelled hit compound and the TMR-labeled derivative with the Grb2-SH2 target derived close Kds for tagged and untagged ligand (Figure 5D). The fact that a higher affinity (Kd) was found for the labeled ligand GhN-11, **2**, most likely indicates an additional small hydrophobic contribution from TMR to the binding free energy.

To investigate whether a purification step introduced between the PS/PS labeling and cleavage of primary on-bead hits and the single molecule assay in solution would improve the quality of the fluorescence titrations and the decoding efficiency, a second screening round with Grb2-SH2 was performed. An additional 20 hit beads with compound affinities between 390 nM and >20 μM were identified (Table S8). The HPLC purification step proved to facilitate the decoding process by MALDI-MS. Eighteen out of 20 samples were successfully decoded. The full scope of the current ICB process can be explored by further taking the resynthesized PS/PS-labeled ligands into cellular validation. To exemplify this, we expanded the PS/PS-labeling strategy by incorporating a bifunctional tag for conjugating both a cell-permeable peptide and a fluorescent label. The best resynthesized hit compound from our α/β peptide library was derivatized with an azide-modified, TMR-labeled TAT peptide to produce a cell-permeable construct **4** (Figure 6). By high resolution fluorescence imaging on the PS03 microspectroscopy instrument the TAT-conjugated compound **4**, was demonstrated to accumulate in the nucleus of A431 cells after 12 hr incubation (Figure 7A). Furthermore, anisotropy images showed a significant higher anisotropy for the nuclear fraction of the probe as compared to the cytoplasm. This indicates a molecular interaction of the heavily positive charged probe with components in the nucleus (Figure 7B).

The ICB process studies were further complemented by a more standard cell biological investigation. The activity of compound **5**, which lacks a label, but contains a Kno homeodomain-derived cell-penetrating peptide (Balayssac et al., 2006), was tested in human endothelial cells. In comparison to a control peptide **6**, containing only the cell-penetrating peptide sequence, the mixed α/β-phosphopeptide **5** led to a concentration-dependent growth inhibition of HMEC-1 and HUVEC cells (Figures 7C and 7D), demonstrating the biological significance of the obtained screening hit compounds.

DISCUSSION

A high screening speed, fast discovery cycle times, the flexibility to generate project-tuned libraries, and the chemical resource efficiency makes on-bead screening a particularly attractive

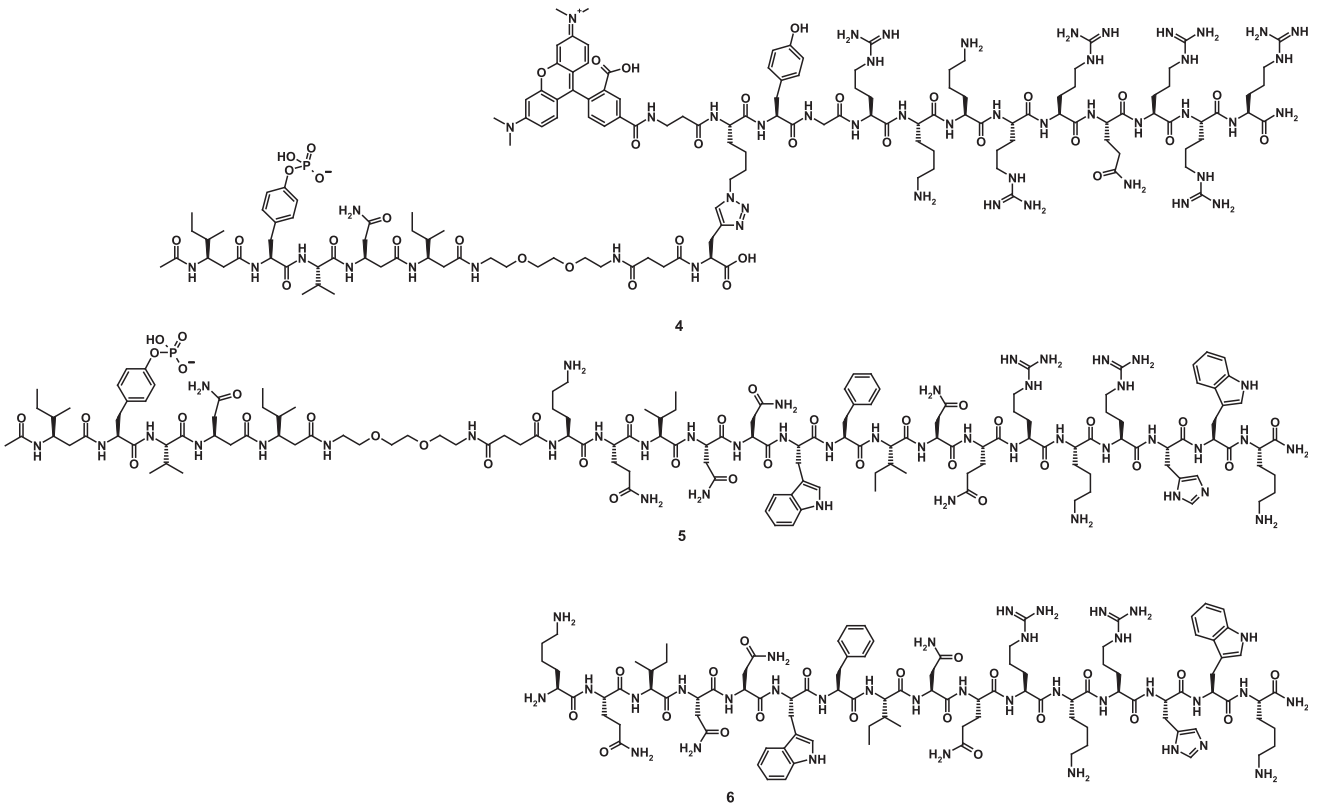

Figure 6. Cell-Permeable Grb2 Ligands

Cell-permeable peptide derivatives of GhT-11 **2** and GhN-11 **3** were prepared for further biological validation. First, a TMR-labeled TAT sequence was conjugated to bead-bound GhT-11 via an ε-azido lysine, using click chemistry to generate the cell-permeable and TMR-labeled derivative **4**. Second, the Grb2-binding sequence of GhN-11 that was C-terminally conjugated via the *ADO* spacer to a Kno homeodomain-derived sequence (Balayssac et al., 2006) generated the unlabelled, cell-permeable derivative **5**. Compound **6**, containing only the cell-penetrating peptide sequence, served as control in functional assays.

ligand identification method, especially for all organizations that cannot rely on large historically grown archives of a million or more compounds. In an ideal screening situation it is expected that the target-compound binding affinity on a bead equals the molecular recognition parameters in solution. Unfortunately, there is rarely a linear relation between a series of quantitative on-bead binding parameters and the respective Kds of the compounds in solution. In the extreme, strong reproducible binding of a target to a compound on bead may result in very low, sometimes immeasurable affinity in solution (Dixon et al., 2006). The limited predictability of a primary on-bead screening result is the central issue of on-bead screening. This becomes a particularly critical issue in a drug discovery program if many compounds are resynthesized that do not live up to the expectations. The scientific reasons for a discrepancy between solid surface and solution binding may well include the following. (a) The target shows affinity to a side product present on the bead. (b) Polar interactions could be more dominant on the solid surface than in solution, leading to a more negative ΔH. (c) Desolvation of nonpolar groups due to the presence of the bead matrix on the solid surface could lead to more positive ΔS. (d) Localization of charged residues, such as arginine, mediate long-range electrostatic interactions. (e) The microenvironment of the bead matrix leads to local pH changes, affecting protein conformation, stability, or aggregation behavior. (f) Reduced mobility of compounds covalently linked to the PEG chains of the bead. All these effects, and probably more, are difficult to neutralize, even by applying a variety of effective blocking agents. The alternative is to establish a direct link between on-bead screening and solution confirmation that overcomes the necessity for resynthesis. With the combination of the two central elements, CONA and PS/PS labeling, we have built a process that allows a stepwise exclusion of false positives and a selection of the most active compounds from a OBOC library for validation in cellular assays. In addition to direct fluorescence labeling of the protein of interest with an organic fluorophore, a number of alternative primary assay formats have been developed and reported in the literature, ranging from direct fluorescence labeling of the protein of interest to enzyme-linked colorimetric assay methods (Lam et al., 1991, 1997; Müller et al., 1996; Olivos et al., 2003; Sweeney et al., 2005; Lehman et al., 2006). Fluorescence-based target protein detection by CONA, however, opens the possibility for recording kinetic data and performing on-bead competition experiments. It is important to note that most predescribed methods are well compatible with applying the herein presented PS/PS-labeling procedure for subsequent determination of ligand binding affinities in homogenous solution.

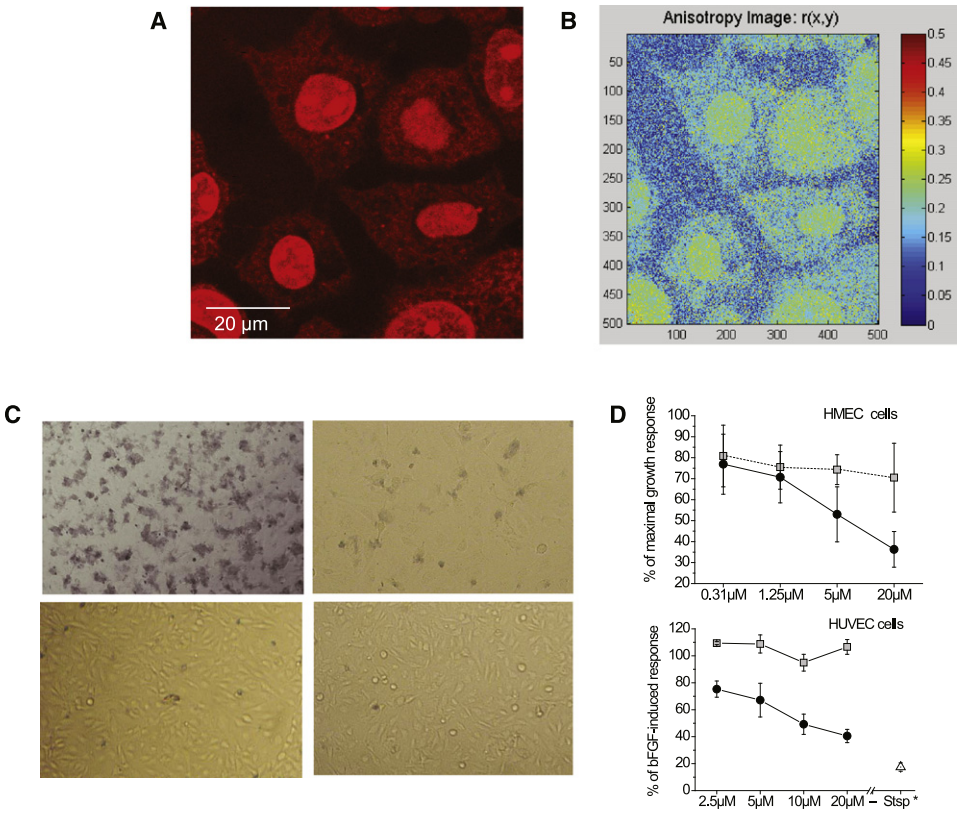

Figure 7. Characterization of Cell-Permeable Grb2 Ligands

(A) Confocal fluorescence intensity image of A431 (human epithelial carcinoma cell line) cells treated with 5 μM of the TMR-TAT-conjugated Grb2-ligand **4** after 12 hr of incubation time, recorded on the PS03 instrument. An accumulation of ligand **4** in the nucleus is detected.

(B) Confocal fluorescence anisotropy image of the same section of A431 cells as in (A).

(C) Effect of cell-permeable Grb2 ligand **5** and control peptide **6** on HMEC-1 (human microvascular endothelial cell line-1) cells (trypan blue staining, 20× images): 20 μM peptide **5** (left top), 5 μM peptide **5** (right top), 20 μM peptide **6** (left bottom), and 5 μM peptide **6** (right bottom).

(D) Effects of peptides **5** (black dots) and **6** (gray squares) at different concentrations on proliferation of HMEC-1 (top) and HUVEC (bottom) cells. The percentage of maximal growth response (cpm values obtained in the absence of peptides) is plotted (data points represent means from four independent experiments ± SD).

The direct solution confirmation represents a strictly quantitative measure for the quality of each ligand derived from each hit bead. The multiparameter confocal fluorescence fluctuation spectroscopy assays used for testing on-bead hits in solution are highly miniaturized (few microliter assay volumes) and inherently sensitive (using 1 nM ligand concentration). It is often argued that a fluorescence label on a small molecule might impact the protein binding affinities. In our experience only small effects of affinity reduction or enhancement of PS/PS-labeled hit compounds are detected compared to unlabeled derivatives. Furthermore, the 2D-FIDA anisotropy assay used for determining the ligand affinity in solution allows immediately spotting of influences of the label on the protein binding affinity. In experiments where the label is involved in protein binding, pronounced effects on the molecular brightness parameter will be observed (fluorescence quenching or brightness increase). By analyzing the molecular brightness changes during the binding reaction, such effects can be identified and taken into consideration for ranking of the ligands. In addition, it is noteworthy to point out that the PS/PS-labeling method provides free choice of label.

SIGNIFICANCE

On-bead screening of large one-bead one-compound libraries can easily result in hundreds to thousands of hit beads. With the high compound density on commonly used micro beads and the thermodynamic differences of ligand-protein binding on the solid surface and in solution, a method for the efficient identification of the best hit beads and compounds is needed. Hence, for a full exploitation of its potential, a bead-based screening process has to comprise automated and quantitative methods for detecting protein-ligand complex formation on bead and for measuring the affinity of these complexes in solution. The screening process reported herein combines quantitative on-bead screening by CONA, with generic PS/PS labeling of the chemical substance on individual hit beads. This tagging scheme allows a direct and reliable affinity determination of primary hit compounds to their protein targets in solution and a stepwise exclusion of optical, chemical, and thermodynamic artifacts prior to resynthesis. Thereby, any follow-up activities can be focused on compounds, with

compelling target binding affinities and specificities. Furthermore, the ICB screening process operates with minimal chemical resources, i.e., the approximately 20–100 picomoles of compound originally contained on individual 90 μm TentaGel beads. The multiparameter confocal fluorescence fluctuation spectroscopy methods used for target binding affinity measurements in solution are highly miniaturized (1–10 μl assay volumes) and inherently sensitive (using 1 nM ligand concentration). Consequently, the amount of compound derived from individual hit beads is sufficient for hundreds of assay points opening up the possibility for broad specificity testing. The fluorescent ligands generated en route provide valuable tools for cellular validation studies and model organism imaging. Looking beyond high throughput screening on bead, the combination of single bead labeling and handling techniques can deliver large fluorescently labeled small molecular libraries for biochemical, cellular, and model organism screening.

EXPERIMENTAL PROCEDURES

For detailed procedures on chemical synthesis, single bead purification, MALDI-MS structure decoding, cell biology, and imaging refer to the Supplemental Experimental Procedures.

On-Bead Screening

For on-bead screening, wells from a 96 well microtiter plate were filled with 1 mg of resin from the individual sublibraries of the phosphopeptide library. The beads were then swollen in PBS and 0.01% Tween20 (200 μl per well, 10 mM phosphate [pH 7.4]) and sonicated for 1 min to break remaining bead clusters. After buffer removal, the beads were treated with 150 μl of blocking buffer at 4°C for 1 hr under constant shaking. Then, a solution of Cy5-labeled Grb2-SH2 protein (50 μl; 20 nM in blocking buffer) was added to the samples and incubated for 8 hr at 4°C. To generate a monolayer of beads the plate was shortly vortexed at ~600 rpm, followed by a sudden stop. This resulted in a homogeneous sedimentation of the beads onto the well bottom. Immediately afterwards the plate was mounted on the PS04 instrument and the confocal scanning started.

Blocking Buffer

PBS (10 mM phosphate, 137 mM NaCl, and 3 mM KCl [pH 7.4]), 0.25% (w/v) gelatine (dry gelatine for blocking buffer; Sigma-Aldrich), 0.01% (v/v) Tween20, and 0.2% (w/v) BSA.

Bead Scanning, Image Evaluation, and Bead Picking

The PickoScreen04 instrument (PS04) is designed for automated scanning of multiple wells from MTP plates. After scanning and image merging of all wells from the screening plate was completed, image analysis was performed using the software package Optimas (Media Cybernetics; version 6.2) to select those wells containing hit beads.

Picking Procedure Steps

As Preparation for picking, a capillary (~2.5 cm length and 140 μm diameter) was mounted onto the picker arm and aligned to the coordinate system of the instrument. All tubings were then thoroughly flushed with water to remove any air from the system.

A rescan of the well from which hits are to be picked was carried out immediately before picking. The image of the rescan was then analyzed by the BeadEval software (version 2.2; Perkin Elmer) and a bead detection performed. The bead detection routine uses the Hough transformation (Ballard, 1981; Thomas et al., 1992). In the bead detection procedure, the xy coordinates of all beads along with their intensity parameters are determined and candidate beads for picking were selected.

The picker arm was consecutively positioned above the xy coordinates of the respective hit beads from the pick list. Picked beads were deposited into 96 well filter plates (Innovative Microplates).

PS/PS Labeling of Individual Hit Beads in 96 Well Filter Plates

Prior to labeling, the beads in the filterplates were washed with methanol using a vacuum manifold (VWR International). After sealing the filter plates at the bottom with a sealing film (Parafilm; 3M), each well was treated with 26 μl of a four-component labeling solution (10 μl H_2O, 10 μl tButanol, 3 μl catalyst solution, and 3 μl dye solution) and finally sealed. The reaction was allowed to proceed under constant agitation for at least 16 hr at room temperature. After removal of the top and bottom sealing, the wells were drained through the filters. The labeled beads were then washed thoroughly with methanol and water, inspected under a microscope, and manually transferred into autosampler glass vials using a micropipette (Gilson; Microman M10).

The dye solution was 2 mM methanolic solution of TMR-azide **1**. The catalyst solution was a freshly prepared mixture (1:1) of ascorbic acid (10 mg/ ml) and copper sulfate (5 mg/ml) in water.

Cleavage of PS/PS-Labeled Compounds from Resin Beads

Labeled beads were treated with an ice-cooled solution (6 μl) of NaOH (1M)/ dioxane (1:1) for 15 min at room temperature. After neutralization with HCl (4 μl, 1 M), the cleavage solution was evaporated under reduced pressure.

Solution Confirmation of Cleaved Compounds by Confocal Fluorescence Fluctuation Spectroscopy

To generate stock solutions the cleaved and dried material from each hit bead was dissolved in 20 μl of acetonitrile (20% v/v) in water. One microliter aliquots from each sample were further diluted 1:500 in PBS (10 mM phosphate, 137 mM NaCl, and 3 mM KCl [pH 7.4]), containing 0.005% Tween20. All solution confirmation measurements were performed in a total assay volume of 7 μl on the PS02 instrument (typical measurement time 12 × 12 s per sample) at ambient temperature, using low volume 384 well microtiter plates (Perkin-Elmer).

Single Point Solution Confirmation and Affinity Determination

Complex formation between the PS/PS-labeled hit compound and Grb2-SH2 was monitored by recording the fluorescence fluctuation data for each compound in the presence and absence of Grb2-SH2 (24 μM) and by determining the fluorescence anisotropy with 2D-FIDA[39]. For an estimation of the significance threshold (i.e., the anisotropy increase due to viscosity changes at higher protein concentrations) identical measurements were performed with free TMR-azide **1**.

For affinity determination, a titration series containing 10 to 12 measurement points of increasing Grb-SH2 protein concentration was recorded. Fluctuation signals for individual wells were recorded in replicates of 12 × 12 s for each titration point.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eleven figures, and eight tables and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00209-9.

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