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High throughput screening of one-bead-one-compound peptide libraries using intact cells

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Abstract

Screening approaches based on one-bead-one-compound (OBOC) combinatorial libraries have facilitated the discovery of novel peptide ligands for cellular targeting in cancer and other diseases. Recognition of cell surface proteins is optimally achieved using live cells, yet screening intact cell populations is time-consuming and inefficient. Here, we evaluate the COPAS large particle biosorter for high throughput sorting of bead-bound human cell populations. When a library of RGD-containing peptides was screened against human cancer cells that express $\alpha_v\beta_3$ integrin, it was found that bead-associated cells are rapidly dissociated when sorted through the COPAS instrument. When the bound cells were reversibly cross-linked onto the beads, however, we demonstrate that cell-bead mixtures can be sorted quickly and accurately. This reversible cross-linking approach is compatible with MALDI-TOF/TOF mass spectrometry-based peptide sequence deconvolution. This approach should allow one to rapidly screen an OBOC library and identify novel peptide ligands against cell surface targets in their native conformation.

Introduction

Recent advancements in nanotechnology have combined targeting molecules with imaging agents and/or therapeutics into a single entity, enhancing their site-specific delivery while reducing off-target toxicities [1-4]. While peptides isolated from biological systems (i.e. phage display libraries) have provided many valuable targeting agents [5-7], they are likely to be susceptible to proteolytic degradation under physiological conditions. For this reason, peptides that are discovered using these approaches can be unsuitable for *in vivo* studies [8]. One-bead-one-compound (OBOC) library screening methods are a chemistry-based alternative to peptide ligand discovery, and have been used previously to identify novel ligands for molecular imaging [3, 9-12], protein inhibition [13, 14] and directed therapy of diseases [15-17]. OBOC libraries are comprised of 90 micron-sized beads each bearing a unique ligand, and can be synthesized using straightforward chemistries [18] and screened in parallel against cell surface targets [19]. The primary advantage of OBOC peptide libraries is the incorporation of non-natural components, such as D-amino amino acids, or the incorporation of cyclic, turned or branched ligands [9]. This facilitates the identification of peptide ligands that are resistant to proteolytic degradation, making them more suitable for *in vivo* applications.

For the purpose of OBOC library screening, the target protein is typically modified with a chemical or fluorescent tag [20-22]. While this approach is feasible for many targets, proteins must be purified and derivatized prior to screening. This increases the risk that these proteins would adopt an altered conformation and could impair their function [23]. In many cases, this approach will not account for changes in conformation due to protein activation. For cell surface proteins and/or proteins that typically form complexes with other cell surface proteins, the presence of the plasma membrane and binding partners may be required for proper folding and the display of biologically relevant epitopes. These limitations can be addressed through the development of cell-based assays to screen OBOC libraries. Indeed, screening approaches using living cells have been successfully utilized to discover ligands against human cancer cell lines including Jurkat T-leukemia [10], T-lymphoma [24], and breast cancer [12]. Nevertheless, conventional methods for isolating rare positive hits from a large OBOC library through manual techniques are inefficient and challenging.

To increase the throughput of library screening, instruments such as the Complex Object Parametric Analyzer and Sorter (COPAS) from Union Biometrica have been employed to isolate high affinity ligands from OBOC libraries using purified target proteins [25-27]. We initially evaluated this platform to sort OBOC combinatorial libraries that had been incubated with living cells, and found that associated cells rapidly dissociated from the library beads once they were passed through the instrument. We hypothesized that by stabilizing the binding of cells to their associated library beads, it would be possible to utilize an automated sorting approach. To this end, we evaluated a reversible chemical cross-linking method to stabilize the association of cells and library beads and assessed the impact on sorting in the COPAS instrument.

An important element of screening throughput is the efficient deconvolution of hit peptides, which we have previously addressed through a MALDI-TOF/TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry (MS) approach [28]. This strategy allows one to perform photochemical cleavage of the peptide from the solid support, followed by transfer of the peptide to the MALDI target, peptide desorption-ionization using MALDI-TOF and sequence determination based on the fragmentation pattern. We were concerned, however, that chemical cross-linking might interfere with accurate sequence determination using mass spectrometry. To test the compatibility of reversible cross-linking with this approach, we performed a library screen using $\alpha_v\beta_3$ integrin-expressing fluorescent cancer cells and a focused OBOC peptide library. Utilizing a library comprised of RGD sequences fused with a representative mixture of amino acids, the cancer cells were cross-linked to the library beads and then sorted using the COPAS instrument. The resultant hits were sequenced using the on-bead MS approach and sequence results were compared between beads that were cross-linked, not cross-linked, or first cross-linked and then treated with heat to reverse the cross-links in order to determine the impact on sequencing accuracy.

Methodology

Peptide library synthesis

Fmoc-based solid-phase peptide synthesis was carried out using an APEX 396 autosynthesizer (AAPPTEC) with 0.05 mequiv of 0.26 mmol/g TentaGel S NH2 (0.27 mmol/g) resin. A threefold excess of Fmoc-ANP and subsequently, the protected amino acids was used in coupling reactions. Fmoc removal was carried out using a solution of 20% piperidine in DMF (N,N-dimethylformamide) over two cycles (10 and 20 min). Amino acid activation was carried out with three equivalents of HBTU and six equivalents of DIPEA (N,N-diisopropylethylamine), which was followed by amino acid coupling over 30 and 120 min cycles. Deprotection of peptide side chains was accomplished using a solution of 88% TFA (v/v) + 5% H_2O (v/v) + 5% phenol (m/v) + 2% triisopropylsilane (v/v) over 6 hrs.

OBOC library screening using live cancer cells

$\alpha_v\beta_3$ integrin-expressing MDA-MB-435 breast cancer cells were labeled with green fluorescent protein. Approximately 1,000 beads containing each peptide were equilibrated with serum-free DMEM in a 12-well plate. MDA-MB-435 cells were detached from the flask by EDTA and resuspended in serum-free DMEM. 200,000 of MDA-MB-435 cells were added into each well containing the library beads and placed in a shaking incubator (50 rpm) for 1 hr at 37^{o}C. The beads were washed twice with PBS, and then imaged under the Olympus IX70 inverted fluorescent microscope. The cells were then fixed onto the beads with 4% formaldehyde for 5 minutes at room temperature, and washed twice with PBS.

Sorting of positive hits using COPAS

The beads from each well were inserted into the COPAS large particle flow cytometer (Union Biometrica), and sorted into a 96 well plate. Firstly, the sorting threshold was established with empty TentaGel beads that have never been previously treated with cells. This step is necessary because TentaGel beads auto-fluoresce, especially in the green (excitation wavelength 488 nm) and red (excitation wavelength 561 nm) channel. The instrument was then gated to only analyze and isolate beads with fluorescence well above the set threshold. This population represents beads that have the strongest association with cells. Any beads with fluorescent intensity higher than the set threshold were sorted into a 96-well plate. Beads that were isolated were imaged under the Olympus IX70 inverted fluorescent microscope. The beads were treated rigorously with ethanol to remove any bound cells, and washed several times with water.

MALDI TOF MS/MS sequence analysis

Cleavage of peptides from TentaGel beads was carried out using UV irradiation. All care was taken to prevent light exposure to synthesized peptides prior to ANP-linker cleavage. For this reaction, approximately 1-3 peptide conjugated TentaGel beads were placed in 200 μL of MilliQ water in an open-top 384 well polypropylene plate. UV irradiation was carried out using a 365 nm UV lamp (UV Products, Upland, CA, model EL25, 8 mWcm−2) over 2 hours. Water was added periodically in order to prevent wells from drying, thus reducing possible peptide decomposition. The resulting peptide-containing solution was then used for MALDI-TOF/TOF analysis.

In a typical experiment, the exact molecular ion mass [M+H]+ of a peptide is determined using MS analysis. MS/MS spectra are subsequently recorded for the desired molecular ion peak, previously observed by MS. This was then followed by manual deconvolution of all peptide sequences in this study.

Results

Sorting OBOC library bead/cell mixtures using COPAS

Large format automated sorters such as the COPAS from Union Biometrica have been used previously to separate positive hits from OBOC libraries based on fluorescence[27, 29, 30]. Given the potential advantages of screening these libraries against live cell populations, we sought to evaluate the ability of the COPAS instrument to sort cell-bound beads. To this end, beads coated with RGD-containing GRGDS peptide were incubated with $\alpha_v\beta_3$ integrin expressing MDA-MB-435 fluorescent breast cancer cells as we have done previously[3]. After passing the bead-bound cell mixture through the COPAS biosorter, the vast majority of the fluorescent cells had dissociated from the beads. Considering the high affinity for $\alpha_v\beta_3$ integrin for its peptide ligand RGD, we concluded that it would be impractical to sort live cell-bead populations using the COPAS instrument without stabilizing the cell-bead interactions.

Cross-linking of live cells and peptide library beads enables automated sorting

The COPAS large format particle sorter utilizes a low flow rate and gentle air-based sorting technique to minimize physical damage to sorted samples. Despite this, we found that live cells were rapidly dissociated from the library beads when passed through the instrument. To overcome this limitation, we evaluated the impact of cross-linking the cells onto the library bead prior to sorting. It was expected that this would prevent the cells from detaching during the fluorescence-based bead sorting process in the COPAS instrument, and peptides with the strongest affinity for cells could then be sorted individually into a 96-well plate (Figure 1). There was some concern that the cross-linking process would impact MALDI MS-based peptide sequence determination, so to test this, a focused peptide library incorporating a GRGDS base peptide sequence and two additional amino acid residues was synthesized. The additional amino acids represented the majority of possible combinations, including GRGDSYT, GRGDSTW, GRGDSWK, GRGDSVP, GRGDSHL, GRGDSFA, and GRGDSPS (Figure 2).

After peptide deprotection, the focused library of RGD-containing peptides was incubated with live MDA-MB-435 cells expressing green fluorescent protein (GFP) (Figure 3a and Supplementary Figure 1). The beads were then washed to remove any unbound cells, fixed in 3% formaldehyde, and then loaded into the COPAS instrument. To distinguish between the bead and cell populations, TentaGel beads or MDA-MB-435 GFP cells alone were evaluated by COPAS (Figure 3b). Plotting by forward and side scatter, the bead and cell populations were largely separated, allowing the discrimination of the bead population (Figure 3b). Final gating and sorting thresholds were defined using a control population of TentaGel beads coated with AGD (negative control) peptide subjected to incubation with cells, fixation and washes equivalent to the experimental beads (Figure 3c). As we opted not to prescreen the library to remove highly autofluorescent beads, up to 12.5% of negative control beads were selected as positive hits (Figure 3c). Microscopy confirmed that this population consisted of beads with abnormally high auto-fluorescence but with no cells bound onto them. These false-positive beads can be excluded using several approaches, including manually identifying and removing them after sorting[30].

While there was significant association of MDA-MB-435 cells with all of the RGD-containing peptide-coated beads, the degree of association was strongly dependent upon the identity of the two C-terminal amino acid residues. MDA-MB-435 cells interacted most strongly with the GRGDSWK, GRGDSPS and GRGDSFA peptides, with 80.3%, 68.1% and 48% of these beads selected as positive respectfully (Figure 3d). The sorted positive beads were then examined using fluorescence microscopy, and all were significantly coated with fluorescent cells (Figure 3a and Supplementary Figure 1). The GRGRDSYT, GRGDSHL, GRGDSTW and GRGDSVP have relatively weaker associations with MDA-MB-435 cells, resulting in much lower sorting rates of 14.6%, 26.6%, 16.2% and 23%, respectively (Figure 3c and Supplementary Figure 2).

Comparison of peptide sequence deconvolution pre- and post-fixation/sorting

Peptide sequences were determined using a previously described MALDI TOF/TOF approach [28] to evaluate the impact of fixation and sorting on the sequence deconvolution. Peptides were fully deprotected using an aqueous trifluoroacetic acid/scavenger cocktail. Since all peptides were conjugated to the resin via a light sensitive linker, they were first cleaved under a UV light prior to sequencing by MALDI-TOF/TOF. Overall, the obtained mass spectra from unfixed and fixed/sorted beads exhibited similar signal to noise ratios and the derived peptide sequences were identical (Figure 4 and Supplementary figure 3). The difference between the expected and observed values (ΔM) from all peptides before fixation was comparable with the ΔM obtained after fixation and sorting. Reversal of the cross-links was also attempted by heating the beads at 60^{o}C for 10 minutes and then 95^{o}C for 15 minutes, however, this resulted in insufficient peptide fragmentation which was inadequate for sequence deconvolution. This demonstrates that cross-linking cells onto beads stabilized their association for automated sorting yet did not negatively impact sequence deconvolution using mass spectrometry approaches. These results suggest that it is possible, through fixation with formaldehyde, to combine cell-based assays with an automated sorting method to provide a viable method for high-throughput cell-based screening of combinatorial libraries.

Discussion and conclusion

Here, we demonstrate the usefulness of a cross-linking method to improve the association of cells and ligand-displaying library beads during automated sorting. A live cell-based OBOC library screening approach can facilitate the identification of high affinity ligands to target proteins in their native conformation. Typically, one would incubate fluorescent target cells with a combinatorial library and allow the cells to associate with beads that display high affinity ligands. Upon cross-linking of the cells onto their associated beads, beads with high numbers of associated fluorescent cells can then be sorted individually into each well of a 96-well plate using an automated sorter. The cross-linking step does not prevent accurate sequence determination using MALDI MS/MS approaches. Indeed, the average ΔM values from test peptides after fixation and sorting are equivalent to those obtained from peptides before fixation, indicating that the accuracy of sequence deconvolution is not compromised upon cross-linking of bound cells to their associated beads. This somewhat surprising observation is likely due to the fact that a majority of the bead surface does not directly interact with the cells, which leaves sufficient uncrosslinked peptides on the bead surface for cleavage and deconvolution. Reversing the crosslinks by heating the beads resulted in insufficient fragmentation for sequence deconvolution, presumably due to breakdown of the peptides under these conditions.

Given that RGD peptides bind $\alpha_v\beta_3$ integrin with high affinity [31-34], we conducted proof-of-principle experiments using a focused peptide library containing GRGDS flanked by two additional amino acids. These additional amino acids were varied to determine whether the cross-linking step will interfere with MS-based sequencing. Cysteine was excluded to avoid the formation of disulfide linkages, while methionine was omitted to avoid oxidation. Isoleucine and glutamine were excluded because they are isobaric to leucine and lysine respectively, making them indistinguishable by MALDI MS. Interestingly, the composition of these two amino acids significantly affect the binding affinity of the peptides to cells (Figure 3c and Supplementary Figure 2), consistent with other studies [35-37].

This method offers a significant improvement in efficiency compared to currently used approaches. Other studies demonstrating the isolation of novel affinity ligands from OBOC libraries using live cells [12, 38] are typically time-consuming and tedious because hit beads must be isolated manually. Using the COPAS biosorter allows the accurate sorting of up to 300 beads per second, making it feasible to complete an entire screen in less than a week. This straightforward approach relies on the direct interaction between the cells and peptide-coated library beads, minimizing the risk of false positives. False positives are typically caused by non-specific interactions that can arise when extrinsic biomacromolecules such as antibodies are incorporated into the screening process as in magnetic-based separation approaches [39]. The specificity of this screening approach can be further optimized by first subtracting out non-specific beads using cells that do not express the target protein, which also can significantly narrow down the number of hits isolated for subsequent analysis. Beads that are not sorted can be collected and recovered by the COPAS instrument and utilized for subsequent rounds of screening.

A significant challenge facing peptide screening approaches is the reduction or elimination of false positives, particularly when sorting by fluorescence using instruments such as the COPAS. Visualization of OBOC library beads by fluorescence microscopy reveals that a substantial population of beads exhibit significant auto-fluorescence, likely due to the intrinsic fluorescence of certain amino acids. Indeed, others have reported that peptides from false positive beads were rich in Leu/Ileu, His, Phe, and Tyr[30]. In our data (Figure 3c), we found that 12.5% of the control beads with no prior exposure to cells were sorted as positives due to high auto-fluorescence. To mitigate this issue during screening, several approaches can be taken beyond merely increasing the stringency of sorting. First, one can optimize the library through a preliminary sort through the COPAS instrument to eliminate highly auto-fluorescent beads prior to mixing with cells. Second, during the screening phase, one can utilize the optional Profiler II software (Union Biometrica) associated with the COPAS to exclude events that consist of a single broad fluorescence peak rather than a grouping of several high intensity peaks that correspond to fluorescent cells. Third, since the false positive beads are easily discerned under a fluorescence microscope, they can be manually excluded subsequent to sorting as others have described[30].

The primary advantage of utilizing a live cell OBOC library screening approach is that the likelihood of identifying ligands that recognize the native conformation of the target protein is substantially increased. This may also be advantageous to screen for ligands against cell surface receptors that adopt specific conformations under certain conditions. For example, a decrease in extracellular pH causes conformational changes in integrins, which facilitate their activation [40]. Additionally, ligand binding regulates the function of several extracellular surface receptors, i.e. G-protein-coupled receptors [41], through the establishment of new conformational equilibrium. This sets the stage for the identification of ligands that are selective for specific protein conformations or activation states. Ultimately, this could be applied to a personalized medicine approach by screening for ligands specific for cells collected from patients (i.e. localized vs. metastatic cancers).

Overall, the ability to screen live cells in a high throughput manner to identify novel ligands will facilitate efforts in molecular imaging and targeted drug delivery.

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Figure legends

Figure 1. Cell-based screening of OBOC peptide libraries to identify high affinity ligands to cellular targets using the COPAS biosorter. Beads incubated with live fluorescent cells are washed, fixed with 3% formaldehyde and loaded into the sorter. Bead hits with strong interactions with cells exhibit high fluorescence and are sorted into a 96-well plate, while beads with few bound cells are excluded.

Figure 2. **Design of focused library of integrin-binding peptides.** GRGDS-containing heptameric peptides were synthesized on TentaGel beads via a photo-cleavable linker. Two additional amino acid residues (indicated in blue) were incorporated into a library to evaluate sorting and MS deconvolution.

Figure 3. Establishing the sorting parameters for library beads coated with intact cells. (a) TentaGel beads containing GRGDSPS peptide incubated with MDA-MB-435 GFP cells (left) and fixed with 3% formaldehyde (right). Beads with the highest association for cells were sorted using the COPAS instrument. (b) Dot plot showing the bead or cell population. EXT represents extinction (measurement of total light scatter), TOF stands for time of flight, and FLU1 represents the green fluorescence intensity. (c) Dot plot showing the cell-bead populations and their sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead (gated from the upper panel). The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. GRGDSPS-TentaGel beads with MDA-MB-435 GFP were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate. (d) The percentage of beads that were sorted by the COPAS instrument for each RGD-containing peptide.

Figure 4. **On-bead MALDI-TOF/TOF MS sequencing of peptide before and after fixation.** MS/MS spectra of H-GRGDSPS-NH_2 before fixing with formaldehyde (left), and after fixing

plus sorting (right). Peptide sequences from both samples were successfully attained. Fragments labeled b_j^a and y_j^a were calculated by complementarity. The average ΔM values are indicated in blue.

Supplementary figure 1. **Cells remained bound onto the RGD-containing library bead after fixation and sorting.** TentaGel beads containing a series of RGD-containing peptides incubated with MDA-MB-435 GFP cells (left), fixed with 3% formaldehyde (middle) and sorted using the COPAS biosorter (right).

Supplementary Figure 2. **Establishing the sorting parameters for the remaining test library beads coated with intact cells.** Dot plot showing the cell-bead population and sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead. The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. RGD-containing TentaGel beads with MDA-MB-435 GFP were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate.

Supplementary Figure 3. On-bead MALDI-TOF/TOF MS sequencing of the remaining test peptides before and after fixation. MS/MS spectra of RGD-containing peptides before fixing with formaldehyde (left), and after fixing plus sorting (right). Peptide sequences from both samples were successfully attained. Fragments labeled b_j^a and y_j^a were calculated by complementarity. The average ΔM values are indicated in blue.

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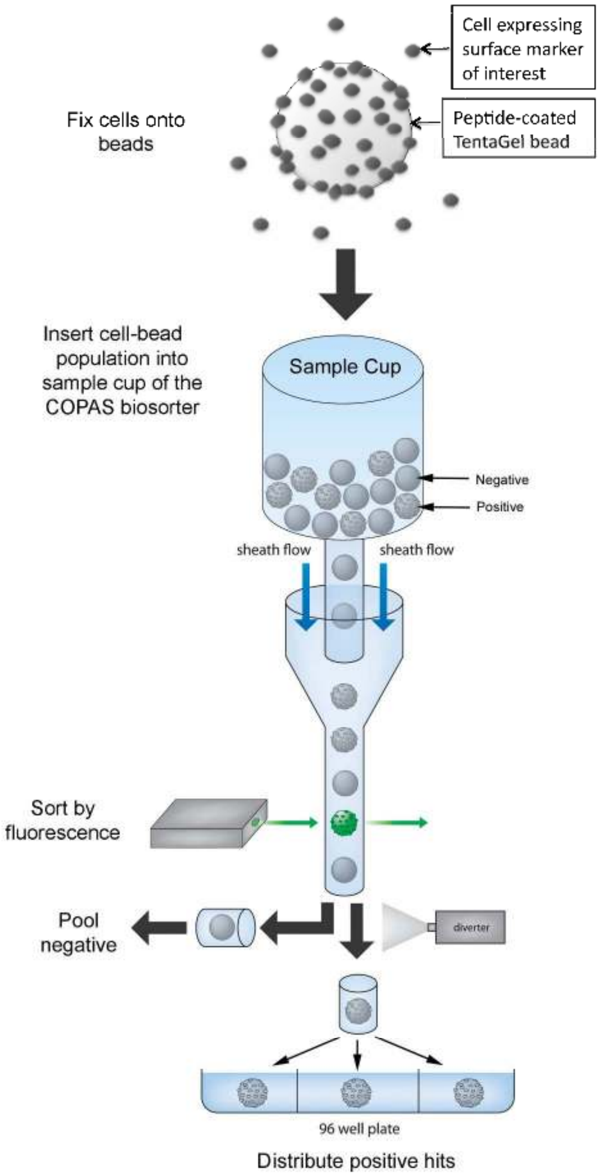

Figure 1. Cell-based screening of OBOC peptide libraries to identify high affinity ligands to cellular targets using the COPAS biosorter. Beads incubated with live fluorescent cells are washed, fixed with 3% formaldehyde and loaded into the sorter. Bead hits with strong interactions with cells exhibit high fluorescence and are sorted into a 96-well plate, while beads with few bound cells are excluded.
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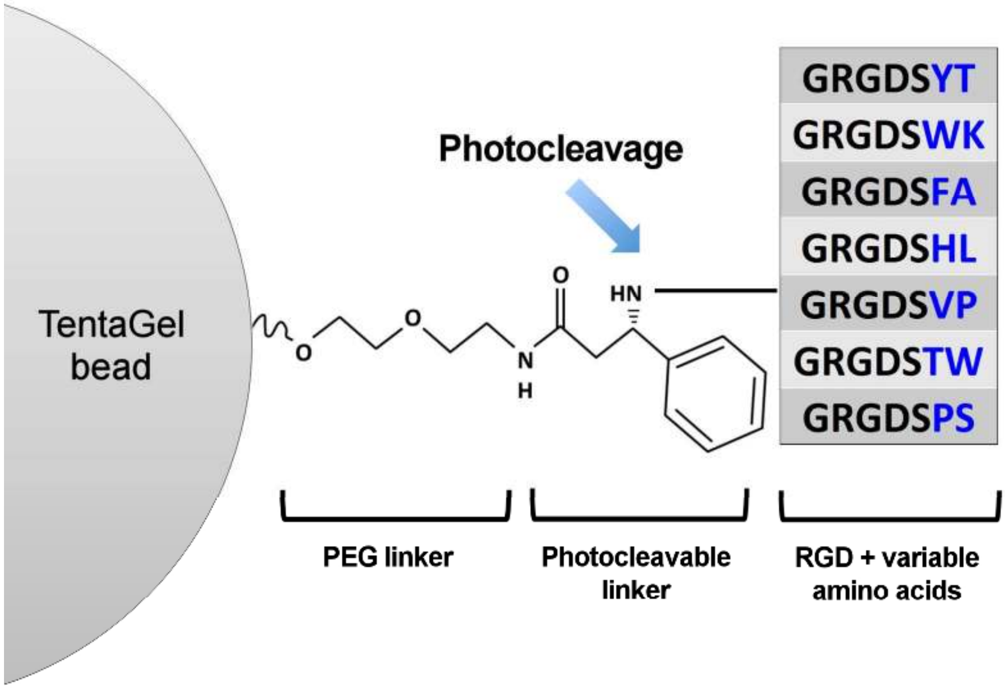
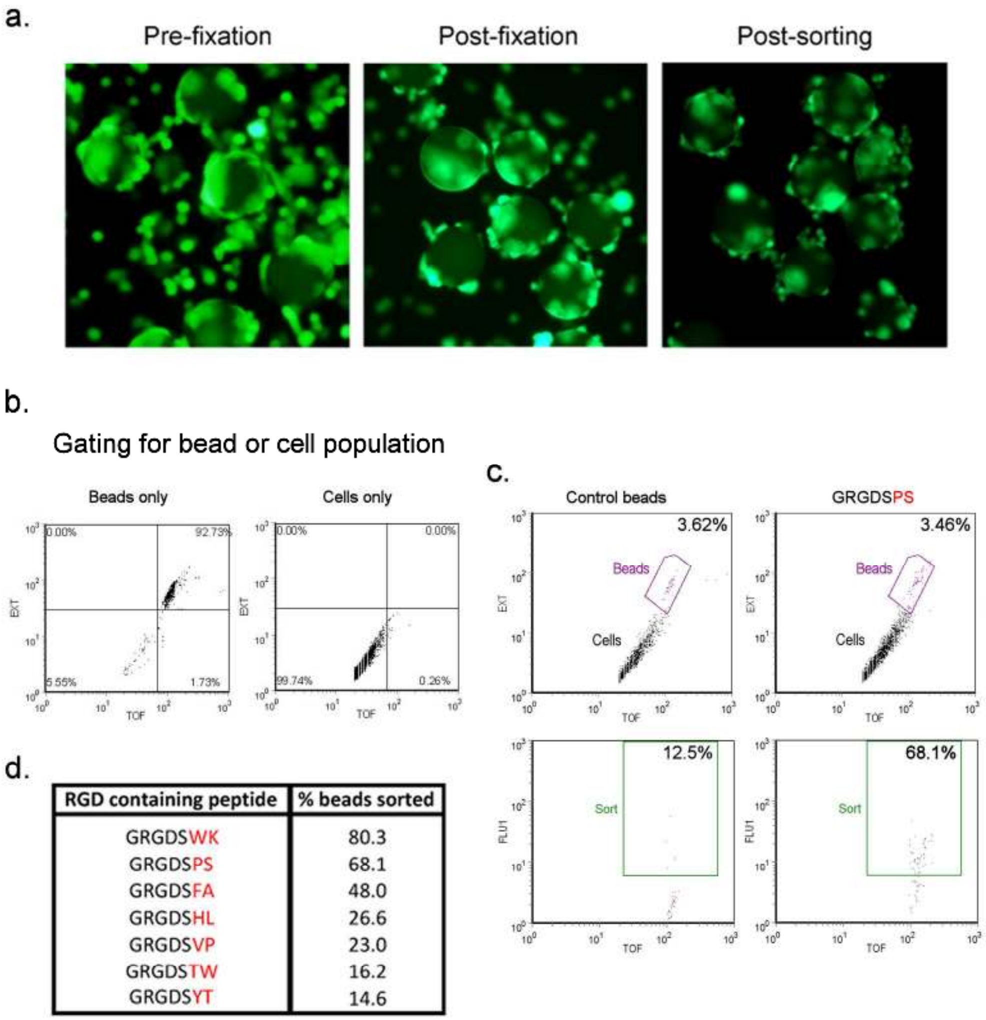


Figure 2. Design of focused library of integrin-binding peptides. GRGDS-containing heptameric peptides were synthesized on TentaGel beads via a photo-cleavable linker. Two additional amino acid residues (indicated in blue) were incorporated into a library to evaluate sorting and MS deconvolution.
148x103mm (300 x 300 DPI)



RGD containing peptide	% beads sorted
GRGDSWK	80.3
GRGDSPS	68.1
GRGDSFA	48.0
GRGDSHL	26.6
GRGDSVP	23.0
GRGDSTW	16.2
GRGDSYT	14.6

Figure 3. Establishing the sorting parameters for library beads coated with intact cells. (a) TentaGel beads containing GRGDSPS peptide incubated with MDA-MB-435 GFP cells (left) and fixed with 3% formaldehyde (right). Beads with the highest association for cells were sorted using the COPAS instrument. (b) Dot plot showing the bead or cell population. EXT represents extinction (measurement of total light scatter), TOF stands for time of flight, and FLU1 represents the green fluorescence intensity. (c) Dot plot showing the cell-bead populations and their sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead (gated from the upper panel). The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. GRGDSPS-TentaGel beads with MDA-MB-435 GFP were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate. (d) The percentage of beads that were sorted by the COPAS instrument for each RGD-containing peptide.
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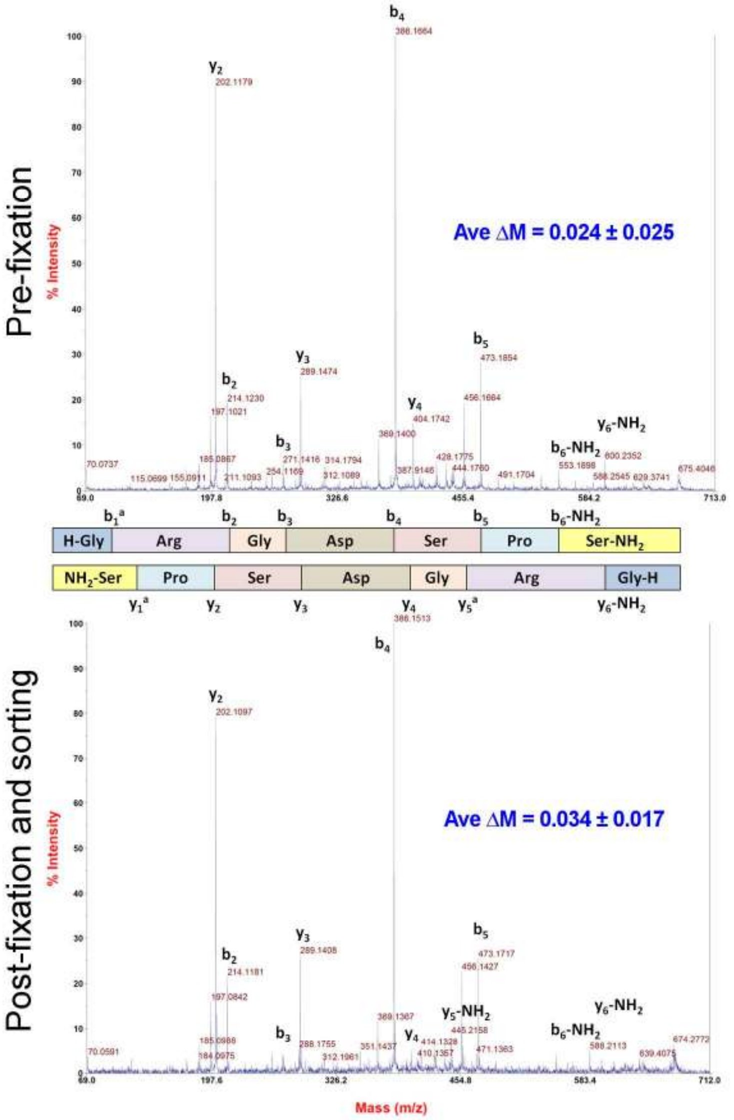

Figure 4. On-bead MALDI-TOF/TOF MS sequencing of peptide before and after fixation. MS/MS spectra of H-GRGDSPS-NH2 before fixing with formaldehyde (top), and after fixing plus sorting (bottom). Peptide sequences from both samples were successfully attained. Fragments labeled bja and yja were calculated by complementarity. The average ΔM values are indicated in blue.
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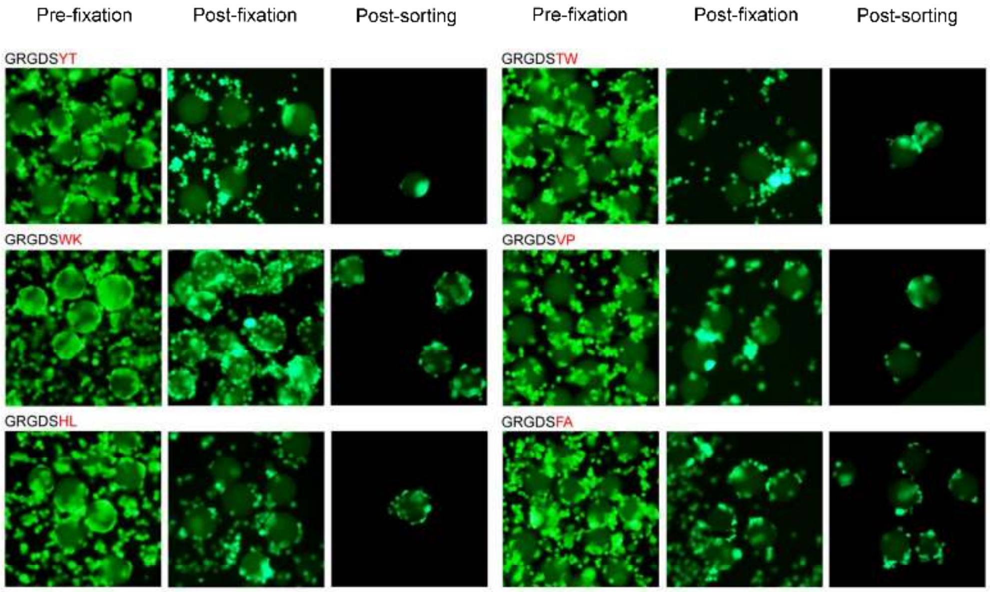
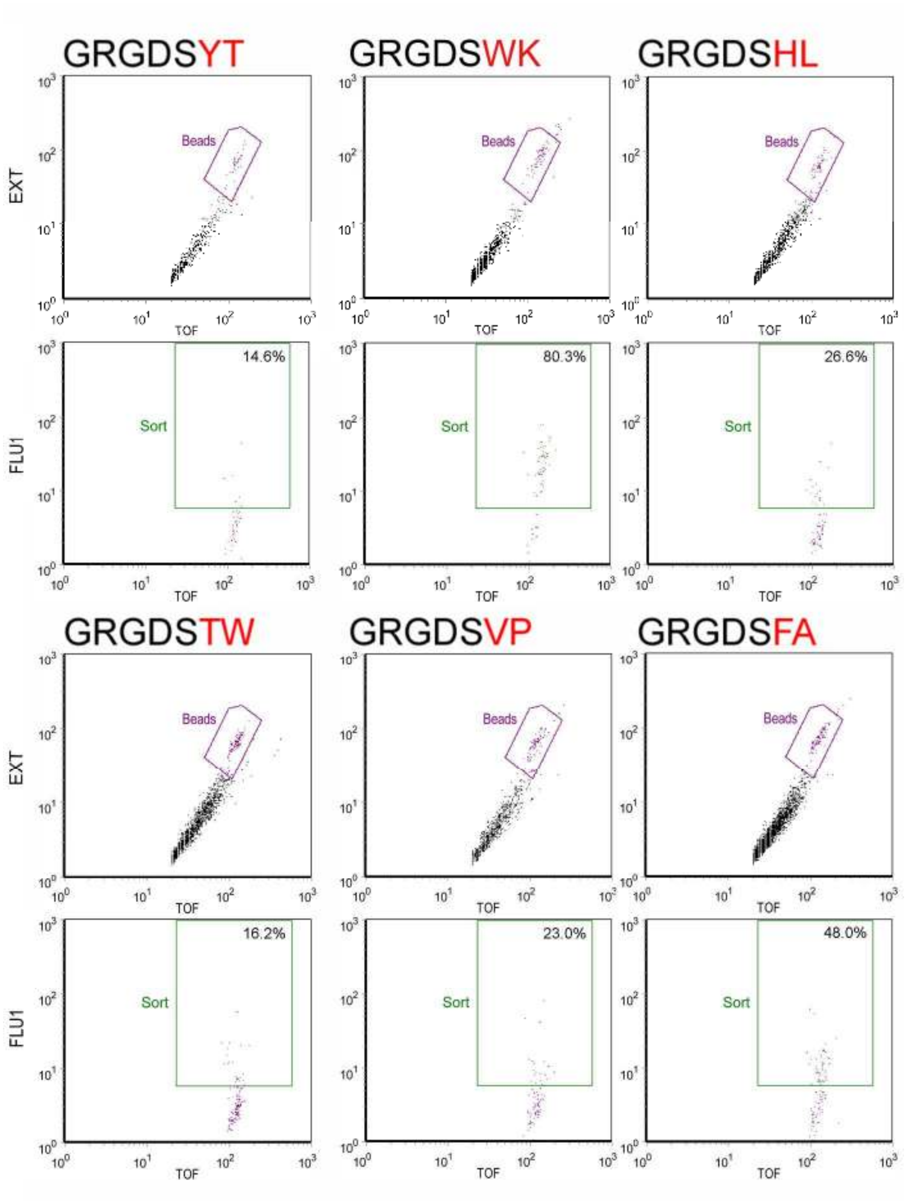


Supplementary figure 1. Cells remained bound onto the RGD-containing library bead after fixation and sorting. TentaGel beads containing a series of RGD-containing peptides incubated with MDA-MB-435 GFP cells (left), fixed with 3% formaldehyde (middle) and sorted using the COPAS biosorter (right).
314x236mm (300 x 300 DPI)



Supplementary Figure 2. Establishing the sorting parameters for the remaining test library beads coated with intact cells. Dot plot showing the the cell-bead population and sorting profiles. The upper panel shows the two distinct bead and cell population (indicated by arrows) and the beads were gated for sorting. The bottom panel shows the green fluorescence intensity of each bead. The sort gate was established using negative control TentaGel beads that has undergone cell treatment, fixation and washes so that only beads with cells attached (higher fluorescent intensities) were sorted. RGD-containing TentaGel beads with MDA-MB-435 GFP were then inserted into the COPAS instrument, and beads with the highest association with cells were sorted into a 96-well plate.
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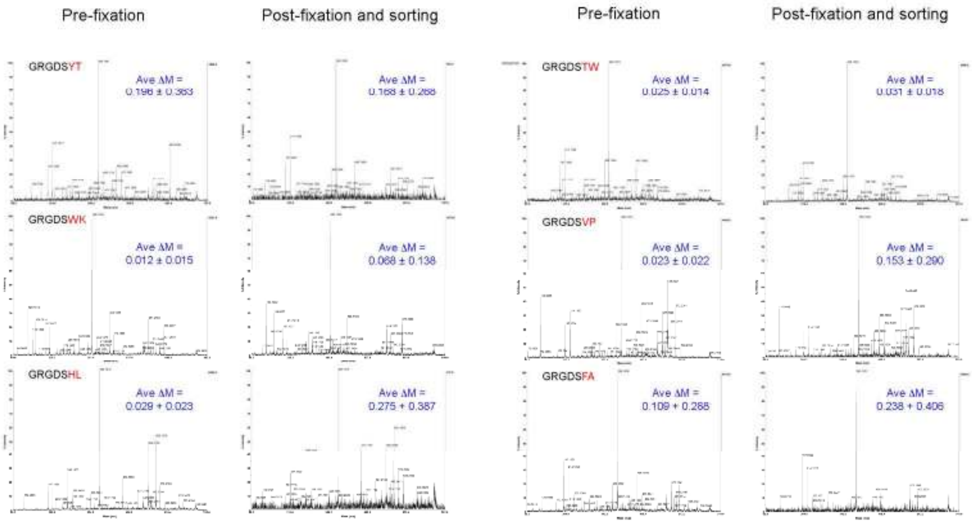

Supplementary Figure 3. On-bead MALDI-TOF/TOF MS sequencing of the remaining test peptides before and after fixation. MS/MS spectra of RGD-containing peptides before fixing with formaldehyde (left), and after fixing plus sorting (right). Peptide sequences from both samples were successfully attained. Fragments labeled bja and yja were calculated by complementarity. The average ΔM values are indicated in blue.
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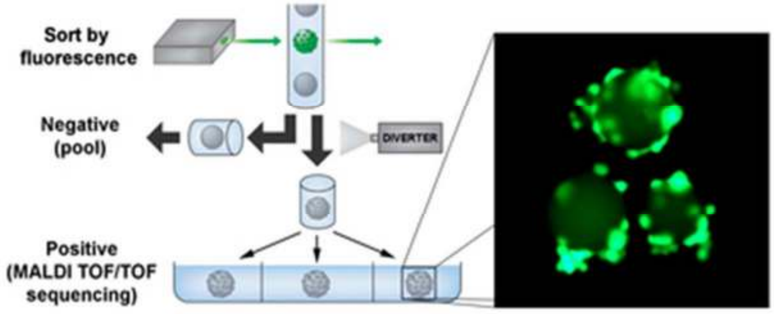

Table of Contents Image:
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Manuscript ID: co-2013-000584
Title: "High throughput screening of one-bead-one-compound peptide libraries using intact cells"
Author(s): Cho, Choi-Fong; Behnam Azad, Babak; Luyt, Leonard; Lewis, John

34x13mm (300 x 300 DPI)