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14. ABSTRACT

Combinatorial library method significantly accelerates molecular discovery and identification in many areas of biology and medicine. Current applied array technique and split-mix approach both have their own limitations. With this view, one-disc-one compound (ODOC) concept was first proposed and aimed to be applied to split-mix peptide library synthesis with the purpose of combining large-scale combinatorial synthesis and digital molecular identification as a whole. The constructed ODOC library may not only overcome the limitation of relatively small library size for array techniques, but substantially reduce the cost and tedious procedure of peptide sequencing for OBOC method through decoding the barcode on the discs. Therefore, the success of ODOC carriers on microfluidic split-mix peptide synthesis may solve the bottlenecks of both array techniques and OBOC method, increase the efficiency of drug discovery and make a potential impact on modern pharmaceutical industries.

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ODOC carriers, barcode, split-mix, peptide synthesis

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Digital ODOC Method for High-Throughput Discovery of Prostate Cancer-Targeting Ligands

Introduction

Combinatorial library methods significantly accelerated molecular discovery and identification in many areas of biology and medicine, e.g., epitope mapping of antibodies, screening of cancer-targeting drugs and recognition of cellular receptors[1-3]. The up-to-date library synthesis strategy of combinatorial methods can be categorized as parallel array and split-mix approach[4-7]. Array technologies can construct high density of molecules in an array format on a solid substrate (microchip), from which the chemical identity of each compound can be directly recognized by recording its location on the microarray. However, these approaches greatly restrict the size of library and its inherent diversity. One-bead-one-compound (OBOC) method one of the most successful split-mix method, invented by Dr. Lam (Co-I) and his colleagues in 1991, has been widely applied for the peptide library synthesis due to its big library size (10^5-10^7) and great efficiency in drug discovery[8-9], however, it suffers from great cost of labor-intensive decoding procedure in order to obtain the sequences of synthesized peptides.

Prostate cancer is one of the most prevailing cancer and the second leading cause of death in Western countries. Up to now, chemotherapy is still the main treatment modality in prostate cancers[10-11], however, the efficacy of the therapy is limited by severe toxic side effects induced by anticancer drugs on healthy tissues. Targeted chemotherapy which can be achieved by attaching a ligand for specific receptors that are expressed preferentially on malignant cells is intended to improve the efficacy of cytotoxic drugs against cancer cells, and meanwhile, reduce toxicity to normal tissues[12]. α6 integrin receptors are cell membrane receptors which have been found closely associated with the progression and metastasis of prostate cancer. Rosca et al. have developed a multivalent α6 integrin-specific construct with three identical peptide segments (-TWYKIAFQRNRK-), which can be used as the targeting probe for the directed delivery of drug or imaging agents[13]. Dr. Kit Lam (Co-I) reported a series of promising D-amino acid peptides with the minimal functional motif of (-kmvixw-), showing specific binding to α6 integrin as well as inhibiting invasion of prostate cancer cells[10, 14].

Microfluidics, in the past decade, has become a pervasive theme in the chemical reactions and biological analyses[15-16]. Benefiting from its miniature systematic dimensions (sub millimeter to micrometer), microfluidic

system has significantly reduced the reagent volume and reaction time. Moreover, the inherent nature of microsystem enables the integration of different functional components in microfluidic system, which further improves the miniaturization and multiplexing of biochemical synthesis and analysis. However, up to now no example of microfluidic platform used for peptide library synthesis has been reported.

To address the above problems, a one-disc-one compound (ODOC) microfluidic method was proposed based on the split-mix principle. It combined the advantages of both array techniques and traditional OBOC method which aimed at combining large-scale combinatorial synthesis and digital molecular identification as a whole. As compared to array techniques, the microfluidic-enabled split-mix method can not only overcome the limitation of relatively small library size, in which the library can be potentially scaled to a size of 10^5-10^7, but also can efficiently achieve split-mix synthesis with microfluidic channels; on the other hand, enabled by the encoded discs (unavailable in OBOC), the sequence of peptide on each disc can be optical readout from decoding the barcode on the disc, which greatly reduce the cost and tedious procedure of peptide sequencing.

To achieve ODOC microfluidic channel-based split-mix method: i) We have successfully achieved large scale fabrication of digitally encoded and amine-functionalized microdiscs; ii) Established microfluidic fabrication and networks for split-mix synthesis; iii) Solved two major problems and made our method a robust method for the peptide library synthesis; iv) We employed the developed ODOC method to successfully synthesize a peptide library with 40,000 sequences; v) Based on the current result, we designed a focused library with the preferred amino acids in each random site and building blocks of LLP2A, this focused library is expected to offer us new insights on cancer-target ligands in a near future.

Keywords: ODOC carriers, split-mix peptide synthesis, microfluidic channels, targeted chemotherapy, α6 integrin receptors

Overall Project Summary

1. Task 1.1 Fabrication of digitally encoded and biochemically activated microdisc carries.

Current objective:

To fabricate and release microdisc carriers using batch machining and to structurally embed recognizable barcodes into each individual microdisc carrier.

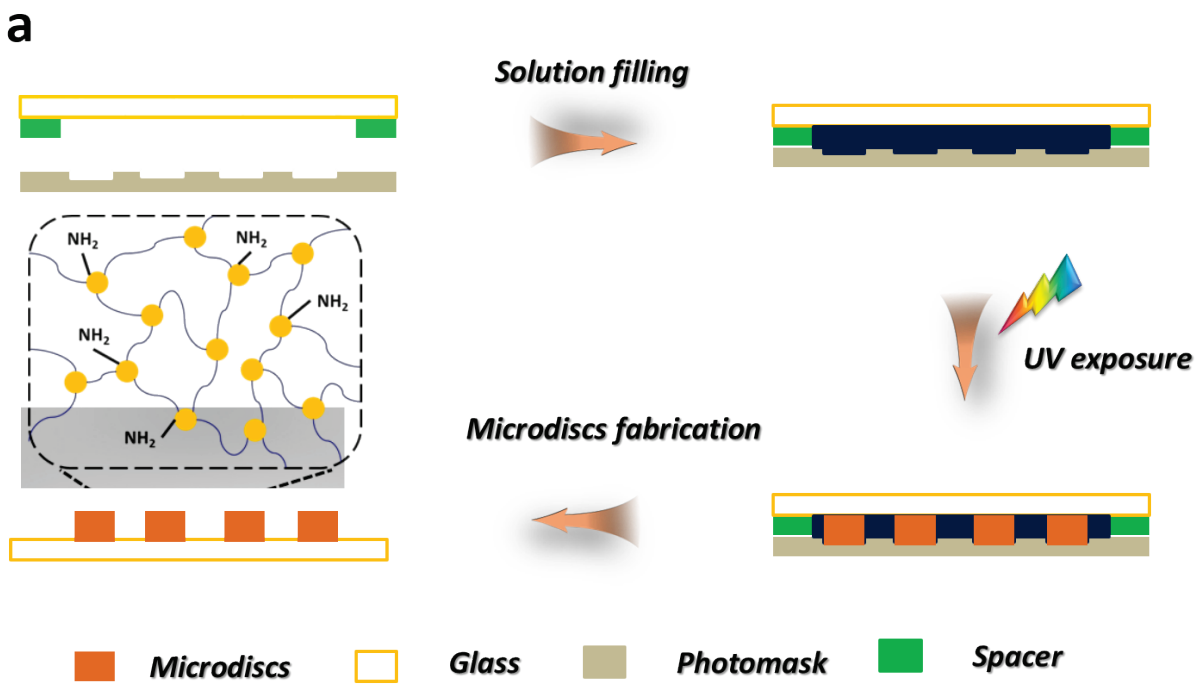
Results and discussion:

The fabrication process for microdisc carriers utilized our newly created microfluidic-based split-mix method was shown in **Fig. 1**. As shown in **Fig. 1a**, the prepolymer mixture was sandwiched between photo mask and glass slide. Microdiscs were fabricated on the glass substrate through polymerization of the prepolymer mixture and the acrylated silane under UV exposure. To achieve the more stable microdiscs for peptide synthesis, the composition of prepolymer mixture was changed to PEG (Polyethylene Glycol)-diacrylate, crosslinker, photo initiator, 2-aminoethylmethacrylate, water, and ethanol (**Fig. 1b-c)**. In this method, PEG microdiscs were fixed onto glass through chemical crosslinking, whereas swelling may cause instability and fracture of microdiscs during peptide synthesis and final TFA cocktail treatment. To build a robust recipe of microdisc fabrication and synthesis, we found that the stability of microdiscs were highly relevant to the thickness of microdiscs, when we reduced the thickness from 8 μm to 3 μm and replaced recipe from A to B, the microdiscs stability was significantly improved but still not consistent. Changing from recipe from B to C, with adding of ethanol, microdiscs undergoing four rounds of peptide synthesis and following TFA treatment became all stable **(Fig. 1c)**, the result can be reliably repeated. Different from the fabrication method developed last year, glass substrates must be silanized first to generate acrylate group on the surface and collection of microdiscs became not necessary.

It is worth-noting that the new method employed photolithography method, so the large area microdisc carriers (10^5-10^7) fabrication can also be achieved. The lithographic resolution of photo-polymerized poly-ethylene glycol (PEG) based co-polymer was also defined (**Fig. 2b**). As shown in **Fig. 2b**, the microdiscs were 110 μm in diameter with 300 μm in center-to-center distance. The size of each information bit is 10 μm and for orientation bit is 10-by-30 μm. We can easily control the geometry of the microdisc carriers by fabrication parameters. The shape and diameter can be adjusted by the design of photomasks. For a carrier with diameter of 100 μm, 34×10^{19} ($=2^{35}$) digital sequences with a minimal lithographic resolution of 6 μm can be encoded. Therefore, through controlling the size of photomask and microdiscs, or repeating the fabrication steps, batch-fabricated array (10^6) of barcoded PEG microdisc carriers with amine-activated surface can be successfully achieved. For example, using a 5×5" photomask, the batch-fabricated array of barcoded PEG microdisc carriers (10^6) with amine-activated surface can be achieved by repeating the fabricating method 6 times.

Similar to the split-mix approach proposed originally, the amine-functionalized microdiscs can also be used for peptide synthesis. As shown in **Fig. 2a**, microdiscs can be stained by bromophenol blue, a widely used dye for

free amine test in peptide synthesis. Before the first amino acid coupling, the microdiscs show the color of dark blue (**Fig. 2a**), while after reaction with L-Fmoc-Val-OH and then stained again by bromophenol blue, there is no blue color appeared on the microdiscs which means that the free amine almost reacted with L-Fmoc-Val-OH. The free amine can be obtained again by piperidine treatment. Therefore, a protecting-blocking-releasing partitioning method, previously reported by Lam's group, can be applied to microdiscs for peptide synthesis.



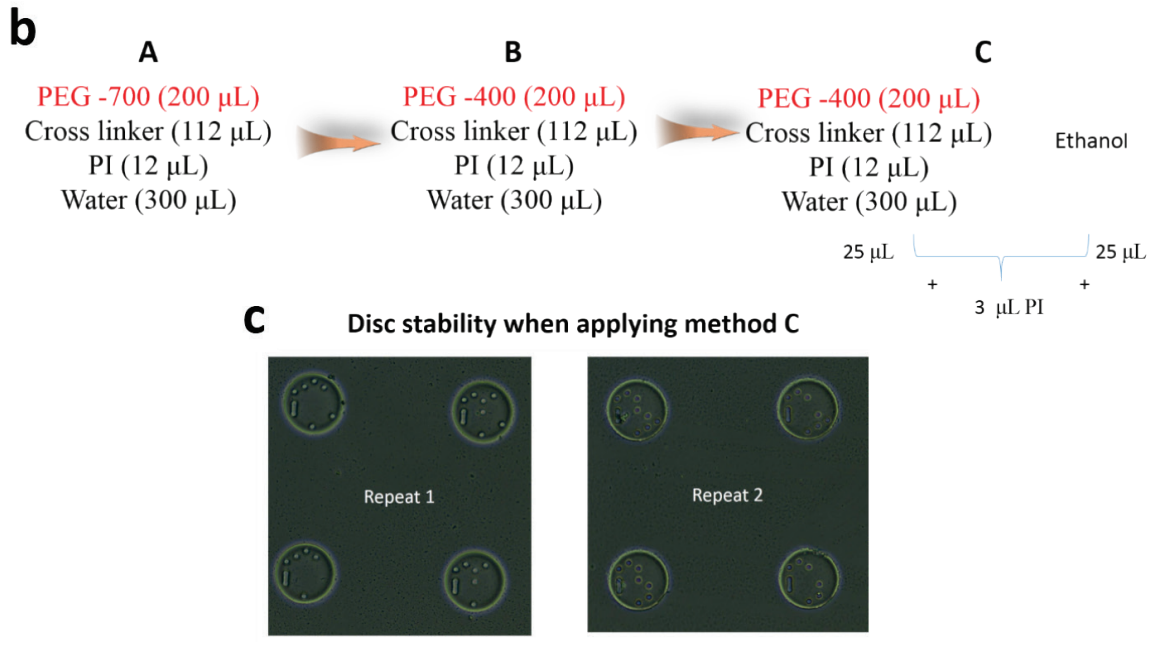


Figure 1. a) Fabrication process for microdisc carriers; b) developed processes for robust microdisc fabrication; c) contrast images of stable microdiscs after 4 rounds of peptide synthesis and TFA treatment.

In brief, utilizing the new method, we achieved a highly efficient microdisc carrier fabrication. Through controlling the size of the photomask, the batch-fabricated array (10^6) of barcoded PEG microdisc carriers with amine-activated surface were successfully achieved. A highly reliable microdiscs with identical geometry were obtained, which formed a solid support for following peptide synthesis. Surface activation of free amine groups on PEG microdisc surfaces can be achieved by employing a protecting-blocking-releasing method.

2. Task 1.2 Encoding and decoding strategies for the digital microdisc carriers.

Current objective:

To get a microscopic view of barcode readouts and achieve a pattern recognizable microscopic picture sets.

Results and discussion:

Similar to split mix method developed last year, a binary encoding scheme was used to design and recognize a distinctive digital-to-chemical identity on each microdisc (**Fig. 2b**). The design of encoding layouts on circular microdiscs also consisted of orientation (the bar indicating the front/backside of the disc) and information bits (the dots encoding unique digital sequences) parts. Replacing peptide sequencing, microscope scanning and recognition of barcode enabled a highly efficient means to establish digital-to-chemical linkage between each numerical sequence and the synthetic identity on the corresponding disc. The barcode scanning system consisted of a microscopic scanning set-up and pattern recognition algorithm. An Olympus IX81 inverted biological microscope equipped with PRIOR H117 motorized x-y stage can be employed to scan and image the barcoded discs. In the next step, the scanned microdisc images can be processed and decoded using a pattern recognition algorithm programmed in Matlab (**Fig. 2b**). Briefly, these microdisc images were first converted to grayscale pictures using object detection functions, from which the outlines and barcode patterns (information/orientation bits) can be recognized. Orientation of each microdisc can be then analyzed by locating its center and the orientation bit from the contrasted image. Subsequently, the information bits were then read and grouped to the corresponding microdisc.

During the past year of research, through the collaboration with Prof. Kwang-Liu Ma's group in Computer Science Department (UC Davis). The algorithm was proven to be capable of read-out current barcode design and convert to digital numerical sequence at a rate of 24 barcodes/ 3 minutes, equal to 7.5 s/peptide, regardless

of the length of peptide on the disc. This algorithm was used to identify barcode on each disc carriers and generated a correlated numerical sequence. This involved recognition of disc boundary, disc rotation adjustment, orientation bit match, information bit setting and sequence output. Since the chemical sequence on each disc carriers was distinct, this created a direct barcode-to-chemical identification. In traditional OBOC method the average time for one amino acid sequencing was 25-30 min, therefore, for a short peptide sequence with 5 amino acids, it often took 2-3 hours to decode. Also with increment of the peptide length, the total peptide sequencing time can further increase. Therefore, with current barcode recognition algorithm, we can successfully achieve a direct barcode-to-chemical identification with much higher throughput (7.5 s/compound) compared with OBOC method.

Overall, a high-speed microscopic barcode-recognition system including both hardware and software were built for the new split-mix method. The design of software of a binary encoding strategy to provide each microdisc with an individually recognizable barcode in a large combinatorial library was constructed. The high-speed microscopic scanning setup which can be used to image individual microdiscs from massive microfluidic assembly in a monolayer configuration was developed[18]. Thus, the high-resolution microscopic images with distinguishable barcodes were achieved which linked the identified microdiscs to the synthetic combinatorial library by using the pattern recognition algorithm.

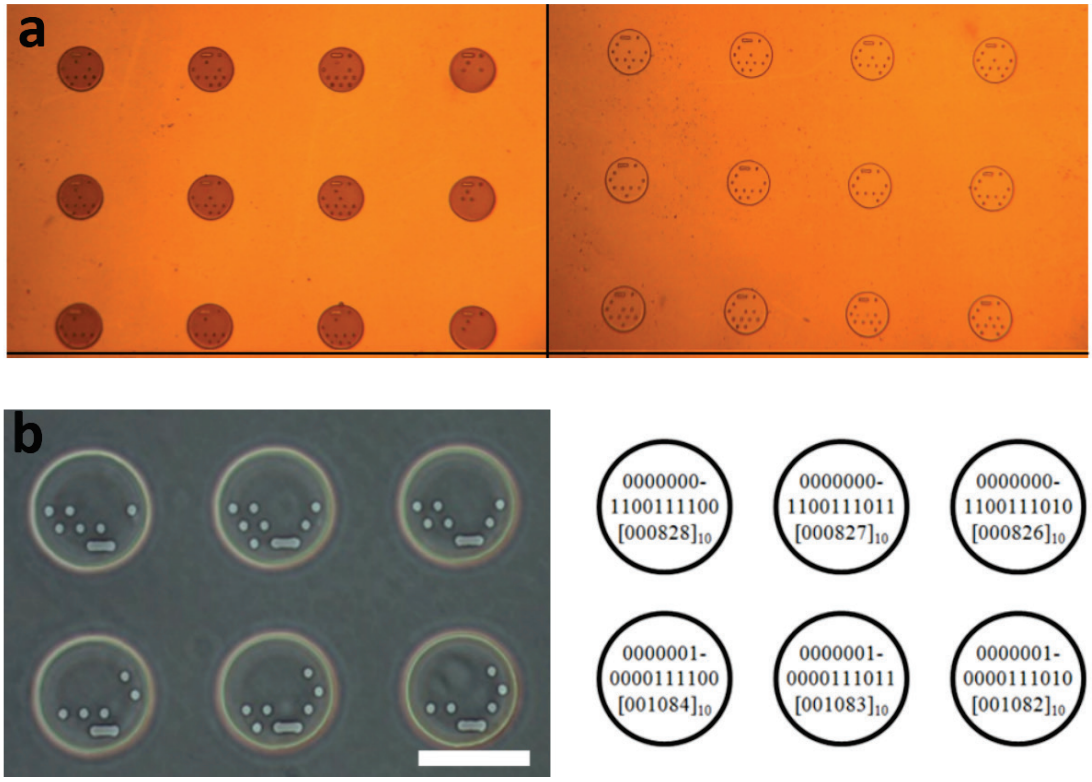


Figure 2. a) Contrast optical images of microdiscs before (left) and after (right) amino acid (L-Fmoc-Val-OH) coupling; b) contrast optical images (left) of barcode on microdiscs and decoding numerical sequences of the microdisc array (right). Scale bar: 200 µm.

3. Task 1.3 Microfluidic synthesis platform with barcode-to-chemical sequence tracking

Current objectives:

A microfluidic synthetic platform was established to adopt the highly efficient split-mix approach while allowing tracking the synthetic history of each element in parallel.

Results and discussion:

Our new developed microfluidic channel based split-mix method aims to build all possible combinations of a given set of building blocks in a minimal number of steps (high throughput) with every molecular structure linked to the digital barcode on the microdisc carrier (addressability). Its mechanism for split mix synthesis can be found in **Fig. 3**. To take -X1X2X3X4 library of three different amino acids (L, D and V) as an example (**Fig. 3a**), as can be seen, the microdiscs on glass can be first reacted to get linker (mix state), then microfluidic chips (**Fig. 3b**) with 'repeating' flow pattern channels filled by L, D, and V coupling solution can be attached

to the glass with encoded microdiscs for the X1 amino acid synthesis (split state). Then removed the microfluidic channels (mixing again) to do the Fmoc deprotection for the whole library. In the second synthetic step, an 'alternating' flow pattern of L, D and V coupling solution was used to add the second amino acid X2 (split), deFmoc (mix), then 'repeating' flow pattern (split) for X3 amino acid synthesis, deFmoc (mix), and finally an 'alternating' flow pattern (split) to synthesize X4 and following operation (mix) again, the whole library with permutations of 3^4=81 was successfully constructed without any omission. Therefore, the microfluidic channel based split-mix method can successfully achieved split-mix peptide synthesis. The new method shows several advantages over old split-mix method. For instance, that microdiscs only needed to be imaged twice before and after cell screening. Through comparing with the pictures and barcode decoding, the peptide on microdisc with high binding affinity can be easily identified. However, in the standard split-mix method, during each synthetic step, every microdisc needed to be imaged and recorded, and the corresponding synthetic building block was then appended to the corresponding barcode sequence.

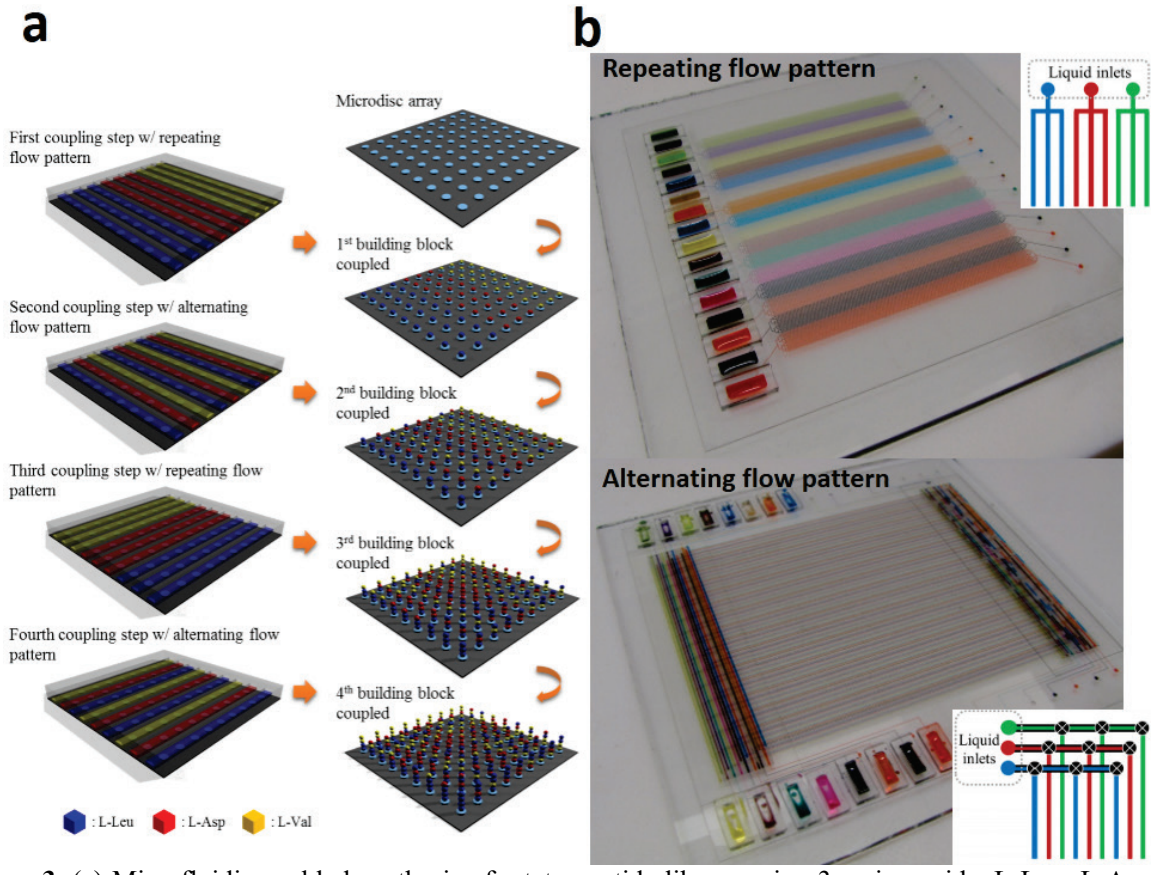


Figure 3. (a) Microfluidic-enabled synthesis of a tetrapeptide library using 3 amino acids: L-Leu, L-Asp and L-Val; (b) synthetic microfluidic patterns and their related design for a tetrapeptide library. The peptide

synthesis procedure was according to the standard Fmoc-strategy used in SPPS. Different colors represent different amino acid building blocks.

To establish the microfluidic-based split-mix method to be a robust method, two major problems should be overcome. One is the instability of microdiscs during peptide synthesis which was solved (**Fig. 1b**). The other problem is the leakage of the microfluidic devices which may induce cross contamination for peptide synthesis. To solve this problem, a covalently bonded PET-PDMS hybrid structure for the microfluidic assembly was developed. As a commonly used material in fabrication of microfluidic devices, PDMS serves as an elastic layer and can enables active devices such as pressurized membranes. However, for structural stability, more rigid plastics were preferred. Therefore, we adopted a plastic-PDMS hybrid architecture, which showed great promises in the microfluidic chip fabrication. To combine PDMS and plastic advantages, a PET-PDMS hybrid device method was developed by completely covalent bonding (**Fig. 4**) which greatly decreases the chance of cross contamination of different amino acids, especially the increased robustness makes the device to be recycle used in whole peptide synthesis. As shown in **Fig. 4**, the plastic was first plasma treated and then silanized to form a layer of hydroxyl groups on the surface. The silanized plastic was plasma treated again to establish hydroxyl group, and then PDMS was spin-coated onto the plastic surface and set to cure, and subsequently, covalently bonded PET-PDMS was obtained. Following the same procedure, PDMS can be spin-coated on the other side of the plastic substrate. With the PET-PDMS hybrid material in place, the bottom layer with inlet can be easily bonded to the device through a standard PDMS-PDMS plasms bonding method. The fabricated device greatly increased the peptide synthesis efficiency and can be repetitively used.

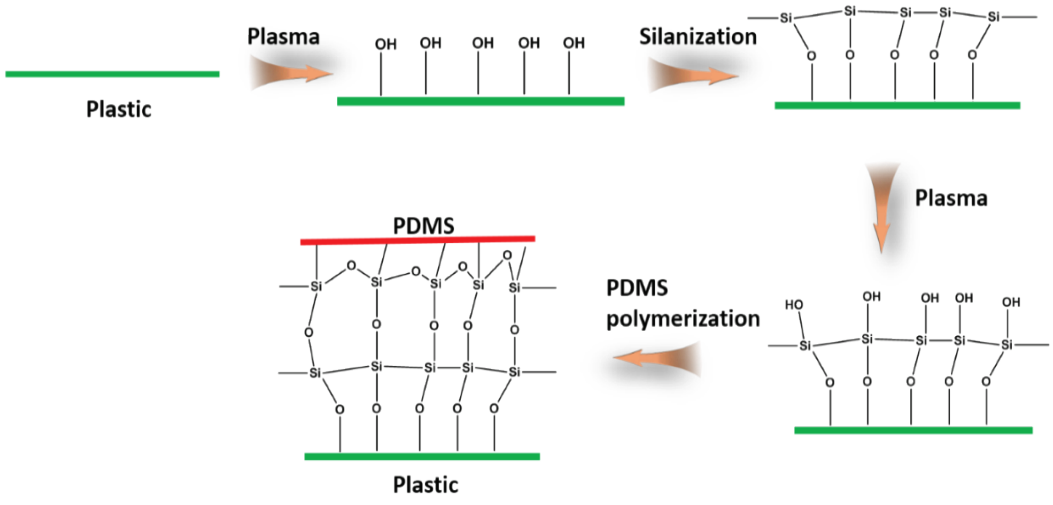


Figure 4. Process of PET-PDMS hybrid material production for further microfluidic chip fabrication.

Recently, the α4β1 integrin receptor has received much clinical interest because of its important role in metastasis and development of lymphocytic leukemia, bone cancer and myeloma. The important function and altered expression level of α4β1 integrin in cancer has made it an attractive target for cancer diagnosis and therapy. Therefore, there is a critical need to identify biomolecules targeting α4β1 integrin with high binding affinity and specificity. To further confirm the accessibility of microfluidic channel based split-mix method for split-mix synthesis, we have tried the peptide library construction. Based on previously published study from Dr. Kit Lam lab [9], we have designed the following tetrapeptide library with N-terminal capped with MPUPA-OH for further improvement on binding affinity and specificity: MPUPA-$X_1X_2X_3X_4$. For each random site (X), we have selected 10 amino acids, every disc has 4 copies, making the total number of permutations of this library to be 4X10^4=40,000.

Follow the synthesis method shown in **Fig. 3**, the corresponding encoded microdiscs library was successfully constructed and used for α4β1 integrin-binding peptides screening on Jurkat cells. The positive peptide sequence on microdiscs can bind to the α4β1 integrin on the membrane of cells which make the cells attached onto the surface of microdiscs. Before the incubating with cells, all the discs were scanned with microscopy, after cell incubation, the discs were scanned again, and the two scanned images were compared to get a chemical binding map which showed the exact peptide sequence through decoding the barcodes on the discs.

The cell binding strength had different dependence on different random positions (**Fig. 5**). As can be seen, cell binding was strongly dependent on the amino acid selection at random positions of X_1, X_2 and X_4. Particularly for X_4, this preference of Asp and D-Glu had never been found previously. Because LLP2A was considered to be the strongest binder so far and only had three position without X_4, our finding provided additional clues for searching stronger binders and their structure-activity relationships in future.

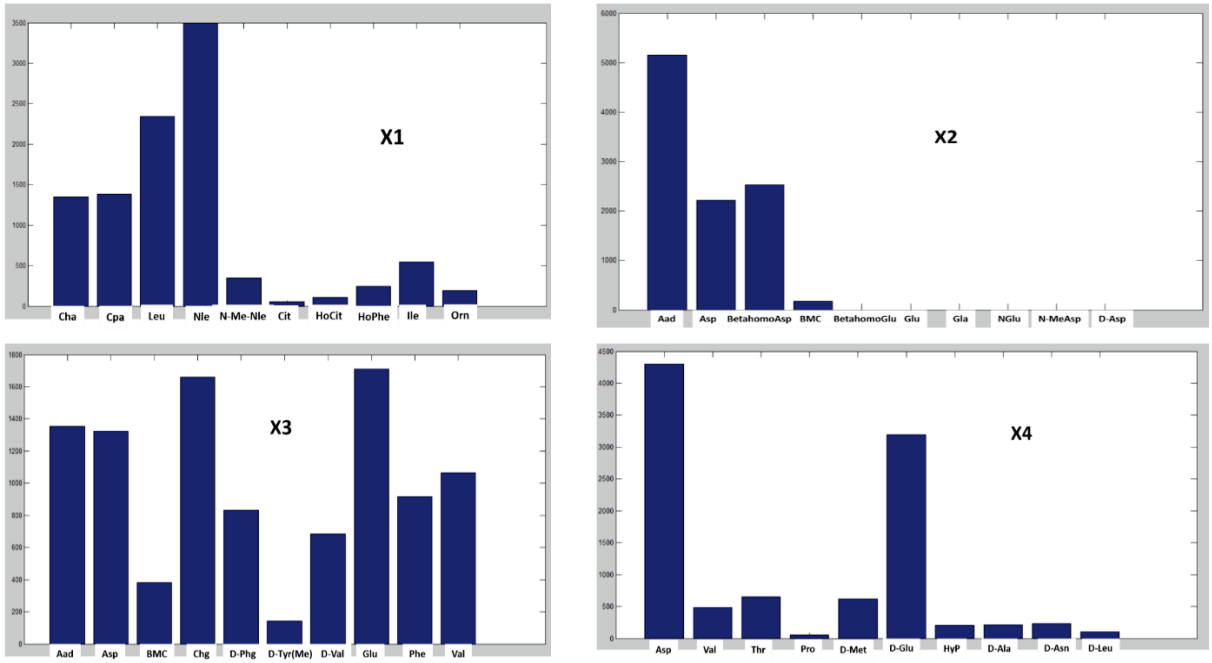


Figure 5. The relative preference value of each amino acid at each random site.

In summary, we have solved major problems and established a robust microfluidic split-mix synthesis method for the peptide library construction. We have developed a new method for plastic-PDMS device fabrication. The new established approach is quite stable and can be repetitively used which greatly increases the peptide synthesis efficiency and quality. We have used the method to successfully synthesize a peptide library of 40,000 members. The preliminary result has showed that the new developed microfluidic-based split-mix method allows to efficiently identify cell-molecular interactions through optical decoding of the barcodes on microdisc carriers.

4. Task 1.4 Preliminary result in design and synthesis of focused peptide libraries

In order to search for a stronger binder, a focus library with 5 copies at different amount of amine was designed, based on our previous results (**Fig. 6**), in which the final library size was 5760. The library contained LLP2A building blocks of ε-6-[(2E)-1-Oxo-3-(3-pyridinyl-2-propenyl)-L-lysine, Aad, Ach. At the same time, the preferred amino acids in every position were also selected. This offered a higher chance to find better binding molecules. In addition, through using Boc-Gly-OH and Fmoc-Gly-OH, the amine amount in every small library can be controlled, which led to different amounts of cell-binding ligands on the surface of discs. Therefore, we can plot the surface concentrations of peptides (as y axis), the number of cell-binding (as x axis),

and then produce a quantitative curve and an IC50 value for each peptide when the binding cell amount reaches half of the strongest binder. In addition, such a value can be used to compare the relative binding affinity of peptides prior to following real IC50 determination. Future research will be concentrated on the focused library synthesis. The peptides found by this method were all new binders, thus future cell signaling (immunohistochemical stain) study would be processed to understand how compound works. It is expected that the new peptide discovery will greatly improve the current α4β1 integrin targeted peptide library.

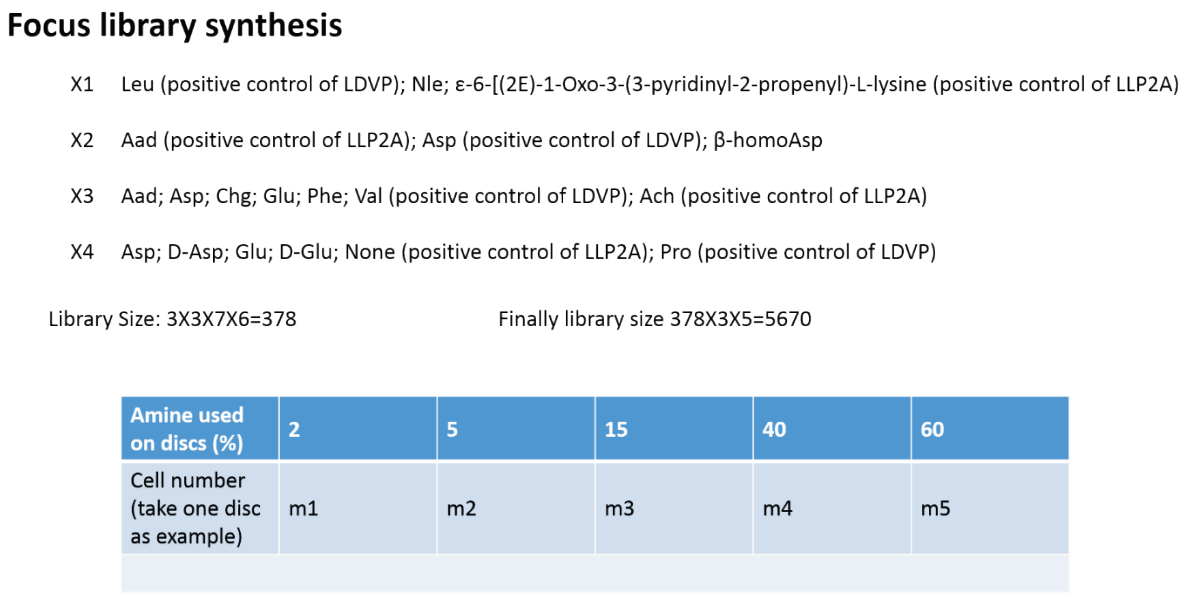


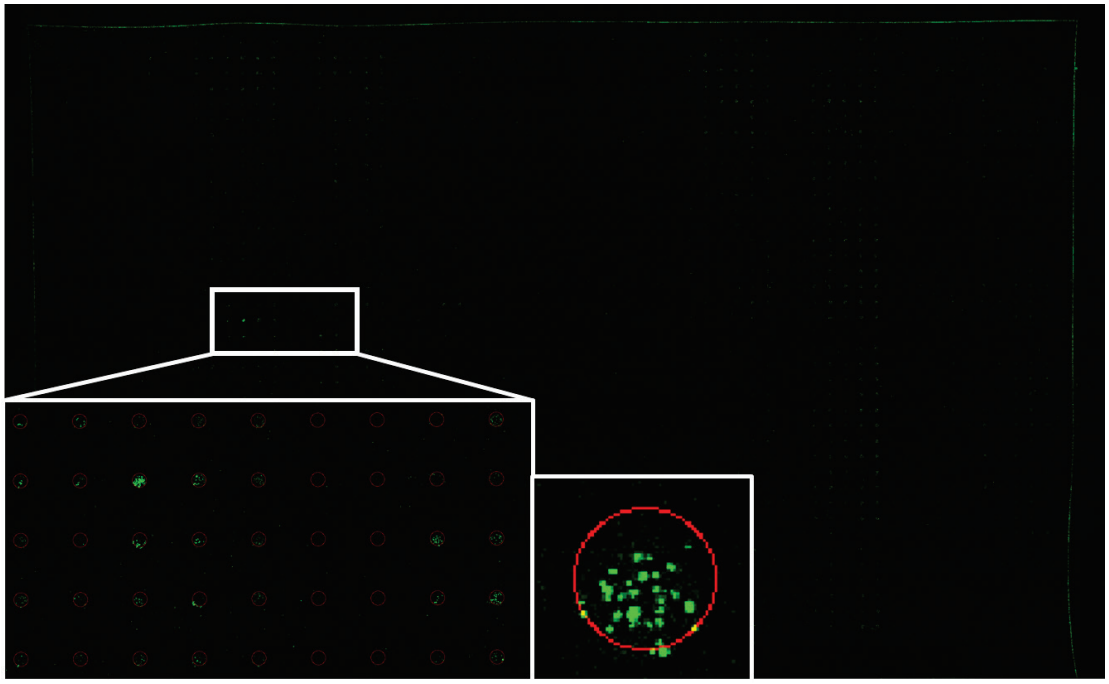
Figure 6. Design of 5 copies of focus library with different amine amount and related amino acids used.

5. Task 2.1 Microfluidic combinatorial libraries for screening of prostate cancer-targeting ligands.

Jurkat cells with luciferase and green fluorescent protein (GFP) are obtained from immortalized malignant T lymphocytic cell line, and maintained in a 95% air/5% CO2 humidified incubator (VWR International, LLC) at 37°C. RPMI (Roswell Park Memorial Institute) 1640, supplemented with 10% FBS (fetal bovine serum) and 1% PS (Penicillin Streptomycin), was used as cell culture medium. Cells were passaged every three or four days at 1 million cells per milliliter density [29]. Upon the experiment, cells were harvested and resuspended to a concentration of 2 million cells per milliliter. The chip with peptide array was then incubated with suspended Jurkat cells in a 95% air/5% CO2 humidified incubator (VWR International, LLC) for 2 h at 37.5 ℃. Extra cells were gently washed off with phosphate buffered saline (PBS) for 3 times, with remaining cells binding to positive sites. The chip was then kept in fresh PBS and examined under a confocal microscope (Zesis).

6. Task 2.2 In vitro and in vivo evaluations of the identified cancer-targeting peptides.

In order to select positive sequences that can target $\alpha_4\beta_1$ integrin within the library, the whole library was screened simultaneously *in situ*. Jurkat cells are reported to have a high level expression of $\alpha_4\beta_1$ integrin on the cell surface [39], and were thus selected as the target for screening the library. After incubation, cell attachment on micro discs was observed. The binding results for the 2560 spots/slide chip was shown in **Figure 7a**, where Jurkat cells are transferred with green fluorescent protein and therefore can be detected under green fluorescent microscope. Each red circle indicates the area taken by one PEG disc. The resulted number of cells binding to each spot was then estimated by customized MATLab program and shown in **Figure 7b**, where z axis is the cell count and x-y plain corresponds to the spatial information of each disc. In order to verify the stability and repeatability of the MPS synthesis method, the same chip was synthesized twice, each with two copies of 1280 library, which ends up with four copies of the whole library. The repeatability was then evaluated by the following steps: first the cell binding strength was divided into weak, medium, and strong binding according to the value associated with cell count on each disc; second, the spots that have identical binding strength level across all different copies were counted; finally the repeatability was estimated by percentage of the identical spots number over total number of spots. Resulted repeatability was calculated to be 91.1% across the four copies, which shows good reliability of the MPS method. Such good reliability also benefits from the compatibility of MPS platform with standard Fmoc chemistry, as known to have very high yield, which is very important for long-chain peptide synthesis. Compared with some other emerging peptide microarray methods (such as light activated synthesis or particle based synthesis), which use different chemistry and possibly result in relatively low coupling yields, the MPS platform offers a more stable and robust way to carry out microarray synthesis.



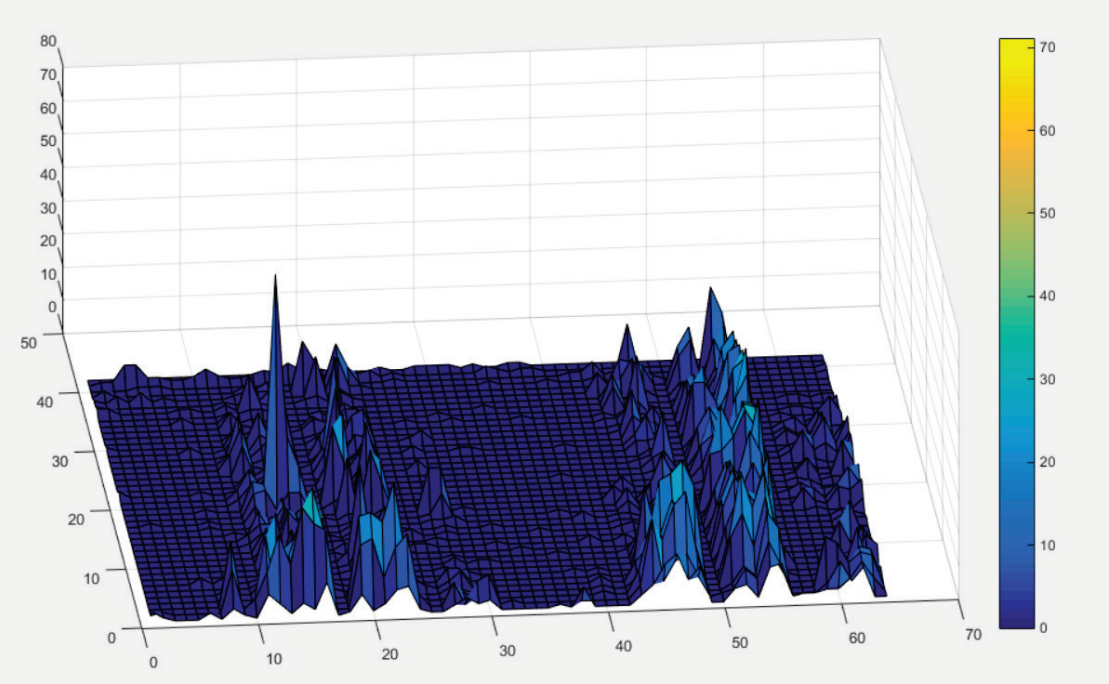


Fig. 7. (a) Cell binding results in green fluorescent channel; **(b)** estimated cell counts for one chip.

The selected binding sequences for the 1280 library was concluded in **Figure 8**, by summarizing the overlapping high binding affinity spots among all copies. From the result some structural related information about the peptide library can be concluded: X_1X_2 positions plays important role in enhancing binding affinity, which result in some strong binding motifs such as Leu-homoAsp-XX, Nle-homoAsp-XX, Leu-Aad-XX and Nle-Aad-XX. The above results show that MPS method can provide much more structural related information for a library, as all sequences at each spot are known. For standard bead synthesis methods [6], only a small number of beads with high binding affinity get selected and sequenced due to high cost of the sequencing procedure, while the sequences for other weaker binding beads remain unknown. With the MPS technique, we can fully acquire the binding results for all sequences in the library, which provides precious information for further analysis and library design.

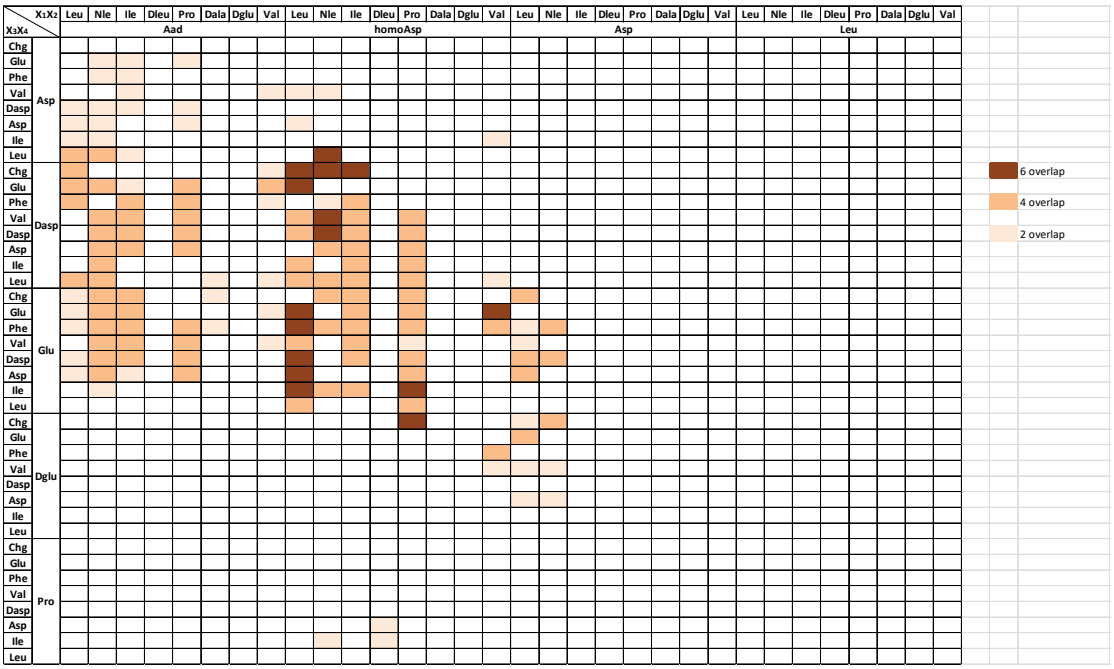


Fig. 8 Repeated binding sequence results for the focused library

Key Research Accomplishments

After completion of the proposed work, we have developed a novel microfluidic-based synthetic method and successfully applied it to the combinatorial library synthesis, including:

For Task 1.1 and related milestones:

i) Minimal lithographic resolution of photo-definable poly-ethylene glycol (PEG) based co-polymer was defined. The microdiscs were 110 μm in diameter and 300 μm in center-to-center distance. The size of each information bit is 10 μm and for orientation bit is 10-by-30 μm. We can easily control the geometry size of the microdisc carrier by fabrication parameters. The shape and diameter can be adjusted by the design of photomasks. For a carrier with diameter of 100 μm, 34×10^{19} ($=2^{35}$) digital sequences with a minimal lithographic resolution of 6 μm can be encoded.

ii) Photolithography and life-off processes were successfully used to batch-fabricate massive arrays of PEG microdisc carriers with structural barcodes embedded. As shown in **Fig. 1**, the pre-polymer mixture was sandwiched between photo mask and glass slide. Microdiscs were fabricated onto glass substrates through polymerization of the pre-polymer mixture and the acrylated silane on the surface of glass under UV exposure. The structural barcodes were embedded in PEG microdiscs, as shown in **Fig. 1c**. Massive arrays of PEG microdisc carriers can be obtained by using a larger size of photomask, improving resolution of microdiscs or repeating the photolithography.

iii) Surface activation of free amine groups on PEG microdisc surfaces can be found in **Fig. 2a**. Before the first amino acid coupling, the microdiscs show the color of dark blue, while after reaction with L-Fmoc-Val-OH and staining with bromophenol blue, there appears no color on the microdiscs, which implies the free amine groups are depleted by the reaction with L-Fmoc-Val-OH. The free amine groups can be recovered subsequently by piperidine treatment for the next round synthesis. Therefore, a protecting-blocking-releasing-partitioning method can be applied to microdisc-based peptide synthesis.

Briefly, Task 1.1 was completely finished. Through the microfabrication of the microdisc carriers, a massive array of barcoded PEG microdisc carriers (10^5-10^7) with amine-activated surface can be successfully achieved.

For Task 1.2 and related milestones:

i) A binary encoding strategy was designed to provide each microdisc with an individually recognizable barcode in a combinatorial library (**Fig. 2b**). The design of encoding layouts on circular microdiscs also consisted of orientation (the bar indicating the front/backside of the disc) and information bits (the dots encoding unique digital sequences). The microdiscs were 110 μm in diameter and 300 μm in center-to-center distance. The size of each information bit was 10 μm and that of orientation bit was 10 by 30 μm.

ii) A high-speed microscopic scanning setup was devised to image individual microdiscs from massive microfluidic assembly in a monolayer configuration. The barcode scanning system consisted of a microscopic scanning set-up and pattern recognition algorithm. An Olympus IX81 inverted biological microscope equipped with PRIOR H117 motorized x-y stage can be employed to scan and image the barcoded discs. Through collaboration with Prof. Kwang-Liu Ma's group in the Department of Computer Science (UC Davis), the algorithm was proven its capability of read-out and convertion from barcodes to numerical sequences at a rate of 7.5 s/compound regardless the length of peptide, which cannot be accomplished by any existing technology.

iii) Individual microdiscs with distinguishable barcodes were identified using a pattern recognition algorithm from the microscopic images. This algorithm was used to identify barcodes on each disc carriers and generate a correlated numerical sequence. It involved recognition of disc boundary, rotation adjustment, orientation bit matching, information bit setting and sequence output. Since the chemical sequence on each disc carrier was distinct, it enabled a direct barcode-to-chemical identification. Such a chemical-to-digital mapping can be found in **Fig. 2b**.

iv) The identified microdiscs was easily linked to the synthetic combinatorial library. The new developed microfluidic-based split mix method showed several advantages over the standard split-mix method, including the fact that microdisc needed to be scanned twice before and after cell screening. Since the chemical sequence on each disc carrier were unique, the peptide on microdisc with high binding affinity can be optically identified, through comparing with the images and barcode decoding strategy developed in ii) and iii).

In brief, a high-speed microscopic barcode-recognition system including both hardware and software was developed. Therefore, Task 1.2 was successfully completed.

For Task 1.3 and related milestones:

i) The newly established microfluidic-based split mix method involved both fabrication of microfluidic channels and microdisc synthesis. As can be seen in **Fig. 3**, the novel method can achieve split-mix synthesis

by using alternating microfluidic channels. In this approach, the reaction chambers were the channels that consisted of the microdisc array.

ii) Split-mix synthesis was successfully achieved by the microfluidic split-mix method. Through scanning microdiscs before and after cell screening by a high-speed microscopic scanning setup, the obtained images were compared and chemical sequences were identified by a pattern recognition algorithm. The data analysis can be found in **Fig. 5**.

iii) Because the microdiscs are all fixed on glass, the scan and tracking the synthetic history of microdiscs during each coupling step was not needed any more. This is also great advantage of this method over old one. Scanning microdiscs was only needed for 2 times, i.e. before and after cell screening.

iv) The microfluidic-based peptide synthesis on microdiscs using standard solid-phase needed to be characterized at the end of each round of amino acid reaction. Microdiscs can be stained by bromophenol blue, a widely used dye to test free amine groups on synthetic surfaces. **Fig. 2a** showed the successful completion of this method.

In summary, we have applied our new developed microfluidic-based split mix to combinatorial peptide library synthesis. Utilizing a high-speed microscopic scanning setup and pattern recognition algorithm, the direct barcode-to-chemical identification was achieved. Such a high-efficiency peptide synthesis approach was applied, from which a 40,000-peptide library was synthesized and screened for proof-of-concept purpose. Therefore, the Task 1.3 was successfully delivered.

For Task 1.4 and related milestones:

i) We have designed focused peptide libraries based on the previously identified potent motif of (-kmvixw-), and incorporate random residues from D- and L- amino acids and unnatural amino acids in the N-terminal and leaving a potential handle for following conjugation with cytotoxic drugs or imaging tags;

ii) We have included an optimization strategy for the identified ligands with high affinity and high specificity to prostate cancer cells from the initial focused peptide libraries.

iii) Based on the initial microfluidic screening results, we have been able to resynthesize the focused combinatorial peptide libraries on the microfluidic synthetic platform using standard solid-phase peptide chemistry;

iv) We have been able to verify the bioactivity of synthesized peptide ligands on microdiscs with cell-line cancer cells. However, we have met with biochemical challenges in this Task, where synthetic outcomes of (-kmvixw-) motif have led to extremely low yield and inconsistent results. Instead of (-kmvixw-) motif, we have continued the library design with more liable chemistry of (MPUPA-$X_1X_2X_3X_4$) motif as our focused screening and as the validation for our targeted combinatorial approach. Therefore, the Task 1.4 was delivered with a change of library design due to the synthetic hurdles.

For Task 2.1 and related milestones:

i) We have been able to assemble a combinatorial peptide library with a massive array of microdiscs into microfluidics;

ii) We have conducted whole-cell binding screening on the microfluidic-assembled combinatorial library;

iii) We are still in process to address the imaging issues in relevance to the differential screening strategy, where a mixture of cancerous and normal cells will be screened under the same microdisc array, so that highly specific ligands can be identified by comparing optical and fluorescent images in a single flow test;

iv) We have performed controlled shear flow tests to quantitatively evaluate binding affinities of massive cell-ligand interactions in parallel on the microfluidic platform.

Under this Task, we have been able to validity whole-cell binding results under the controlled shear flow test. However, due to the imaging challenge we currently have, we are still in process to combine cancerous and normal cells to be screened under the same microdisc array with one imaging setup. Therefore, the Task 2.1 was successfully delivered besides the differential screening strategy is still under way.

For Task 2.2 and related milestones:

i) We have re-synthesized the lead compounds, identified and decoded from the microdisc carriers expressing high affinity and high specificity to prostate cancer cell lines, into both free and biotinylated forms;

ii) We have in vitro determined binding affinity of the re-synthesized α6 integrin-targeting peptides with live prostate cancer cells using fluorescence-activated cell sorting analysis;

iii) We have in vitro evaluated the cytotoxic activity of the optimized ligands against prostate cancer cell lines using standard MTT assay and anti-cell proliferation assay;

iv) We are currently in preparation to use near-infrared imaging to evaluate in vivo the tumor targeting properties of the prostate cancer ligands on xenograft models, from which in vivo efficiency of targeted delivery of optical probes to tumor and normal tissues can be experimentally determined.

Under this Task, we have been able to carry out all the in vitro tests based on our screening results, using the standard binding affinity and cytotoxic activity tests. However, we currently have, we are still in process to use the advanced near-infrared imaging to conduct in vivo animal evaluation. Therefore, the in vitro testing of Task 2.2 was successfully completed while the in vitro study is expected to be accomplished in a near future.

What opportunities for training and professional development has the project provided?

Five graduate researchers and post-doc scholars have been mentored in part on the grant over the award period, Siwei Zhao (Ph.D., 2013), Jiannan Li (Ph.D. student), Zhongliang Li (M.S., 2015), Gaomai Yang (Post-doc), and Wenwu Xiao (Post-doc). Siwei contributed to the initial idea and mathematical modeling of microfluidic synthesis and screening of the peptide library, Jiannan continued working on the microfluidic synthesis and screening of the focused peptide library, and Zhongliang assisted the Ph.D. students and post-docs to conduct those experiments. Gaomai contributed to the microfluidic synthesis and screening of the combinatorial peptide library, and Wenwu focused on in vitro and in vivo evaluations of the identified α6 integrin-targeting peptides.

Research Mentorship to Women and Minority Graduate Students

At the University of California, Davis, a campus with a long tradition of commitment in promoting diversity and equal opportunity, I have been actively engaged in recruiting women and underrepresented engineering students to my interdisciplinary research group, since the start of my academic career in 2006. In particular, I have been promoting an inclusive research environment and mentoring style to graduate students with different backgrounds. Among all 20 graduate students mentored or being mentored by me, seven are female, and one is under-represented (made up 40% of my current/formal group members). Importantly, many of them have been recognized for their excellence in academic and research performances by receiving a number of competitive scholarships, fellowships, and departmental and college awards, including Howard Hughes Integrating Medicine into Basic Science Fellowship, Biotechnology Fellowship, CBST Fellowship, iCAN Contest 2nd Award, Transducers Foundation Travel Award, Glaucoma Foundation Travel Award, and etc. Furthermore, in the campus-wide GREAT program I have organized, we have paid special attention to recruiting and training female engineers and scientists from our international partner institutions, and getting them better prepared for an academic career. Approximately 45% of our total trainees are females.

Summer Research Training to CBST Underrepresented Summer Interns

I consider that the interdisciplinary nature of my research can provide students with exceptional training in relevant aspects of microfluidics, nanofabrication, bionics, nanomedicine, interfacial sciences, and lab-on-a-chip systems. Research activities designed for undergraduates and high school students will promote gifted minority students into the field of nanoengineering and biomedicine. As strong proponent of such educational activities at UC Davis, I have been served as a long-term participating faculty mentor to an internship program in the NSF-funded Center of Biophotonics (CBST) at UC Davis. The CBST summer internship program focuses on recruiting underrepresented high-school and undergraduate students in the greater Sacramento area to interdisciplinary research on campus and the admitted students will be provided with 8 to 12 weeks exposure to cutting-edge biomedical research experience in the mentor's lab. My lab continues to provide three to four internship opportunities to these underrepresented students each summer. It is worth noting that I have recently received the "Outstanding Service Award" from UC Davis Extension for various education and outreach activities I initiated and promoted.

How were the results disseminated to communities of interest?

Introduction of Microfluidics into K-12 Education

As an enabling nanotechnology tool, microfluidics has demonstrated its value in accelerating biological and clinical discoveries. Introducing the principles and functions of microfluidics to K-12 education would be of importance to attract young talents to the emerging nanotechnology fields in the future. The challenge is to present an effective educational solution to the high-school students, which is interesting, playable and also associated with the underlying scientific principles.

I have initiated an outreach effort – Introducing Microfluidics into K-12 Education – to address the unmet educational demand. Recently, my group has developed a plug-and-play microfluidic packaging scheme, known as Microflego – 3D Microfluidic Assembly, to facilely establish complex 3D microfluidic networks using modular building blocks. Besides its potential in large-scale microfluidic integration and packaging, the Microflego assembly can be extended to academic education of microfluidics in a regular wet laboratory setting, where the entire chip can be basically assembled in amateurs' hands with sufficient care, analogous to the renowned Lego® building bricks. Importantly, the reversible Microflego assembly offers an appealing reconfigurable and re-routable potential to switch fluidic functions, which provides an excellent option to illustrate various microfluidic principles and to establish multifunctional microfluidics on demand in a classroom setting. Extended from the Microflego reconfigurable packaging, I am developing a plug-and-play microfluidic building set with multiple functional modular pieces, including diffusion, mixing, pumping, gradients and hydrodynamic focusing modules, which can be easily assembled and demonstrated in a high-school lab setting.

The initial K-12 demonstration is in collaboration with physical science instructors in Mira Loma high school in a Sacramento neighborhood. I along with the lead graduate student plan to train and help the high-school teachers set up the demonstration kit prior to the lab. Participation and organization of interactive lab sessions in a K-12 environment are also included as part of our plan. With the detailed protocols and visual instructions, the latest advances of modular microfluidic concepts would be expected to allow those who are interested in the technology yet with limited resources to explore the field on their own.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to Report

Impact:

What was the impact on the development of the principal discipline(s) of the project?

Overall, the newly established microfluidic-based combinatorial screening method has proven to be successful for synthesis and screening of combinatorial peptide library. Using this method, a large-array fabrication of digitally encoded and biochemically activated microdisc carries can be easily achieved with direct barcode-to-chemical sequence linkage. The encoding and decoding strategies for the digital microdisc carriers have been developed. Based on the our microfluidic combinatorial screening method and findings, some structural related information about the peptide library can be concluded: X_1X_2 positions plays important role in enhancing binding affinity, which result in some strong binding motifs such as Leu-homoAsp-XX, Nle-homoAsp-XX, Leu-Aad-XX and Nle-Aad-XX. The above results show that MPS method can provide much more structural related information for a library, as all sequences at each spot are known.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Nothing to Report

Products

Publications, Abstracts, and Presentations

1. Y. Ding, J. Li, W. Xiao, K. Xiao, J. Lee, U. Bhardwaj, Z. Zhu, P. Digiglio, K. Lam and T Pan†, "Microfluidic-enabled print-to-screen (P2S) platform for high-throughput screening of combinatorial chemotherapy", Analytical Chemistry, 2015, 87, 10166-71.
2. J. Li, G. Yang, Y. Ding, S. Zhao, R. Liu, K. Lam, and T. Pan†, "Droplet based solid-phase synthesis enabled by microfluidic impact printing for large-scale peptide library", manuscript in preparation.
3. S. Zhao, Z. Bai, K. Lam, and T. Pan†, "Microfluidics-enabled Combinatorial Peptide Library for High Throughput Screening," Proceeding of MicroTAS Annual Conference 2014, 1506-1508.
4. S. Zhao, Z. Bai, K. Lam, and T. Pan†, "Digital One-Disc-One-Compound Array for High-Throughput Discovery of Cancer Cell Surface Targeting Ligands," 7th Annual Spotlight on Junior Investigator Cancer Research mini-Symposium, May 13, 2013, Davis, California.
5. J. Li, Y. Ding, W. Xiao, K. Xiao, J. Lee, U. Bhardwaj, Z. Zhu, P. Digiglio, K. Lam and T. Pan†, "High-Throughput Print-to-Screen (P2S) Platform for Combinatorial Chemotherapy", Proceeding of IEEE Transducers Conference 2015, 2236-2239.

Website(s) or other Internet site(s)
Nothing to Report

Technologies or techniques
We have invented a microfluidic-based combinatorial synthetic and screening technology for throughput discovery of cancer-targeting ligands.

Inventions, patent applications, and/or licenses
Nothing to Report

Other Products
Nothing to Report

Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name	Tingrui Pan
Project Role	PI
Research Identifier	N/A
Nearest person month worked	1
Contribution to Project	Lead and oversee the project
Funding Support	

Name	Kit Lam
Project Role	Co-PI
Research Identifier	N/A
Nearest person month worked	0.5
Contribution to Project	Contribute to the combinatorial library design and in vitro and in vivo evaluation of the identified cancer-targeting peptides
Funding Support	

Name	Jiannan Li
Project Role	Graduate Student
Research Identifier	N/A
Nearest person month worked	1
Contribution to Project	Contribute to the microfluidic synthesis and screening of the focused peptide library
Funding Support	

Name	Gaomai Yang
Project Role	Post-doc
Research Identifier	N/A
Nearest person month worked	3
Contribution to Project	Contribute to the microfluidic synthesis and screening of the combinatorial peptide library
Funding Support	

Name	Wenwu Xiao
Project Role	Post-doc
Research Identifier	N/A
Nearest person month worked	7
Contribution to Project	In vitro and in vivo evaluations of the identified α6 integrin-targeting peptides
Funding Support	

Appendices

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