Novel concept microarray enabling PCR and multistep reactions through pipette-free aperture-to-aperture parallel transfer

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Abstract

Background: The microarray has contributed to developing the omic analysis. However, as it depends basically on the surface reaction, it is hard to perform bulk reactions and sequential multistep reactions. On the other hand, the popular microplate technology, which has a great merit of being able to perform parallel multistep reactions, has come to its limit in increasing the number of wells (currently, up to 9600) and reducing the volume to deal with due to the difficulty in operations.

Results: Here, we report a novel microarray technology which enables us to explore advanced applications, termed microarray-with-manageable volumes (MMV). The technical essence is in the pipette-free direct parallel transfer from well to well performed by centrifugation, evading the evaporation and adsorption-losses during handling. By developing the MMV plate, accompanying devices and techniques, generation of multiple conditions (256 kinds) and performance of parallel multistep reactions, including PCR and in vitro translation reactions, have been made possible. These were demonstrated by applying the MMV technology to searching lysozyme-crystallizing conditions and selecting peptides aimed for Aβ-binding or cathepsin E-inhibition.

Conclusions: With the introduction of a novel concept microarray (MMV) technology, parallel and multistep reactions in sub-μL scale have become possible.
screened at once, meaning that each clone can be in a very small amount (less than \( \mu L \)) and highly parallel (more than thousands) and multistep operations are required [9-12]. In other words, the conventional micro-plate (96- and 384-wells) technology, though it allows us to operate in multisteps, requires not a tiny amount of reagents (more than 10 \( \mu L \) or so) if we consider the high degree of parallelism. Therefore, technologies dealing with less than \( \mu L \) aliquots have been pursued. Although there are not a few studies on dealing with sub-\( \mu L \) solutions [13-15], they are basically microfluidics approaches and are currently limited in the degree of parallelism and the number of possible reaction steps [16]. This may be because they are adopting, in a sense, a closed system so as to prevent from evaporation-loss of a tiny volume of sample, leading to rather elaborate and complicated systems. Another approach to earn the multiplicity and smallness of samples is the beads-based one [17] and the essence of it has been successfully adopted in the machine of giga-base (next-generation) sequencers [18]. However, in this approach, there is a difficult problem to cope with: multi-parallel and multistep reactions require the compartmentation of beads. This has already been challenged by some scientists [19]. We made a quite different, unique approach for this challenge: open well type compartmentation.

In this stream, we have devised a multi-well type microarray (open system) made of plastics (dry) and gel (wet) and developed its operational method through pilot experiments such as an arrayed-colony formation, a parallel monitoring of various conditions (for crystallization), and an application to multistep reactions. To develop such technologies, fabricating microarrays in-house was actually prerequisite. As is well-known, the conventional microarray technology has been developed based on the highly sophisticated photo-lithographic technology developed for the IC industry [20]. Therefore, the technological framework was well-established and rather easy to attain a high density of arrays (e.g., millions per square inches). Though the IC-derived technology is good at surface reactions, this technology developed for handling ‘information (state)’ but not ‘materials (molecules)’ has a limitation in being applied to a bulk reaction of molecules. One typical inconvenience is, as shown in Fig. 1, in the inability to succeed individual reaction products at each spot for the next reaction independently, thus making the parallel multistep reactions impossible. Since almost all reactions in vitro need to be multistep ones, a number of separate tubes (or their collective form termed as microplate) have been traditionally used with an aid of robotics, resulting in a massive system [21-23]. Currently, the number of wells per micro-plate has boosted up to almost the limit of 3456 to 9600 with increase of difficulties in operation time and throughput accuracy [24].

In this paper, a novel method depending on a wet and dry microarray system was developed, which enables parallel operations of sub-\( \mu L \) aliquots by overcoming difficult problems such as measuring and transferring sub-\( \mu L \) solutions in parallel without critical evaporation and adsorption-losses of samples. As a result, multidifferent conditions were formed for crystallization of proteins and multistep reactions were successively performed in parallel beginning with PCR followed by transcription, translation, modifying enzyme reaction, and protease-activity assay. Thus, this paper reports a great potentiality of a novel concept microarray MMV for the first time.

**Results and discussion**

First, we need to describe how to make and handle the novel microarray MMV and then proceed to its applications.

**Introduction of microarray with manageable volumes (MMV): how to make and operate**

Following the methods described in Methods, the MMV was prepared and operated (see Fig. 2). MMVs were fabricated using the apparatus built in-house which is composed of DMD (Digital Multi-mirror Device) and others (Fig. 2a) [25,26]. We could form any type of vessels, filters, templates, and others made of either plastics or gel through light-induced polymerization by controlling the light pattern generated by DMD (Fig. 2b-d). MMVs made of gel (wet type) were first introduced here and used in combination with plastics MMVs (dry type) to fulfill multistep reactions as described in Methods. The basic operation of sample transfer from one to another MMV is an aperture-to-aperture way as shown in Fig. 3a and Fig. 3e. In order to demonstrate the feasibility of multistep reactions in the MMV, DNA encoding green fluorescent protein (GFP) was PCR-amplified using an MMV (see ‘MMV operations’ in Methods and Additional file 1). The amplified DNA molecules were transferred to another MMV, partly filled with a solution for the transcription/translation reaction, and then subjected to the reaction. The resulting checker pattern of fluorescent GFP proteins (see Fig. 4) confirmed the success of the parallel aperture-to-aperture transfer and the series of reactions: PCR, transcription, and translation which finally generate fluorescent GFPs (see ‘Verification of the MMV transfer operation’ in Methods).

Without using pipettes, each well of an MMV can be set to be differently conditioned (‘multi-conditioning’) by using various forms of sample-supplying MMVs (Fig. 3c and Fig. 3f). In other words, applying a sample to a particular well can be fulfilled by making the corresponding well chargeable as shown in Fig. 3f. On the other hand, solutions in each well can be transferred to a facing well
in the opposing MMV in parallel, if necessary, separating magnet-bead-trapped molecules from the remaining solvent and solutes as shown in Fig. 3d. Therefore, combining these operations, samples in each well can be independently manipulated at will without using pipettes, which is one of the most remarkable features of the MMV technology. Since handling of ~0.1 µL or less aliquots cannot evade from the instant evaporation problem during the ejection of them from a microsyringe, this feature is valuable in addition to the rapid transfer. Besides, 1000-fold parallelism is another great problem in handling such a minute volume of solution even if
robotics could be employed. This has been a common challenge for MEMS (mechano-electro micro system) and μTAS (micro-Total Analysis System) approaches [13-15,27,28], but yet not completely solved. Therefore, a direct transfer, aperture-to-aperture (Fig. 3e), system is an effective solution for this problem.

To make this system working, both wet and dry MMVs were necessary to be used compensatively: wet one is required for PCR and cell-cultivation while dry one can be used repeatedly for the other purposes such as exchange of solutions. This complementary usage of wet and dry MMVs enabled us to perform multistep reactions which contain molecule-amplification (PCR) reactions as shown later.

Application to a crystallization condition test
To generate multiple conditions (256 species), ‘$2^N$ method’ was employed using dry MMVs. In case of generating 256 different conditions, 8 species of MMV plates ($2^N = 256$; then $N = 8$) were used for this purpose (see Fig. 3f and Fig. 5). The transfer was carried out by spinning two face-to-face stacked MMV plates, a recipient MMV (bottom) and a donor MMV (top) (Fig. 3e). Using the MMV thus made, we could obtain four distinctive types of crystals: typical, micro, needle-like and amorphous crystals (Fig. 6) corresponding to the phase diagram in Fig. 7. This result informs us that the conditions of the lower NaCl concentrations (0.2~0.4 M) and the pH of around 4~5 were especially suitable for obtaining a large tetragonal crystalline...
Figure 3 Basic sample-handling procedures.  
(a) Digital (discrete) addition of samples into MMV wells.  
(b) Initial filling an MMV with sample solution. Aero-bubbles are removed by centrifugation.  
(c) Two ways of regulatory replica-transfer: template way and filtering way.  
(d) Magnetic separation of beads. During the centrifugation, magnetic beads can resist against falling owing to the magnetic force.  
(e) Replica-transferring of samples into recipient MMV wells.  
(f) Template MMVs (T0~T7) used for selective introduction of samples. These 8 patterns of MMVs can generate 256 species of different conditions (see Fig.5).
and that the higher salt concentration (0.9~1.5 M) and the lower pH (pH 3~4) conditions generated the needle-like crystalline whereas the other conditions led to poor generation of crystallines. Such systematic information on crystallization conditions must be very useful for the physicochemical study of morphogenesis mechanism [29-32]. By applying this method, the cost-performance can be greatly improved due to the tiny scale (sub-μL) of this method and, otherwise unavailable experiments must be made possible (see ‘2N Method’ in Methods).

**Figure 4 MMV applications.** (a) Trial version of MMV handling. DNAs encoding the GFP gene charged in checker-patterned wells are PCR-amplified there and then transferred to another MMV where the transcription and translation experiments were performed, resulting in the expression of fluorescent GFP proteins. The variation in the fluorescence intensity came from rather weak controlling of transferred volumes (not completely eliminating lateral (side) transfer during the transfer by centrifugation), which should be conquered in future. (b) Photograph of a checker-patterned MMV. Well patterns of an MMV can be freely designed as described in Fig. 2d. For the sake of clarity, we chose a checker pattern to verify the transfer operation. Here, an MMV made of acrylamide gel is shown.

**Figure 5 2N method.** Each well on an MMV can be addressed using the binary number. For example, in case of 16 wells-MMV (16 = 2^4, N = 4), address-allotting becomes as shown in the center of this figure. If we regard each order (bit) corresponding to a different element (in this case, color), then 4 different (orthogonal) elements need to be prepared. If we take ‘1’ as ‘add’ and ‘0’ as ‘not add’, each binary addressing corresponds to directing which elements should be added there. So, the template way (see Fig. 3c) requires 4 different template plates as shown in this figure. By using these four templates, 16 different states can be prepared in an MMV.
Figure 6 Lysozyme crystallization. In 256 different conditions, four distinctive types of crystals: typical, micro, needle-like and amorphous crystals were obtained. Some of the wells of an MW are shown as inset (close-up images).

Figure 7 Phase diagram of the crystallization of lysozyme. Four distinctive types of crystals were obtained under the various ionic strength and pH conditions. Each state of crystallization is shown: yellow (typical crystal), green (needle-like crystal), blue (micro-crystal), gray (amorphous one), and white (undetected).
Introduction and effectiveness of the wet MMV  
In addition to the plastic MMV made of PDMS (polydimethylsiloxane) or acrylate resin as a dry type, we originally introduced a wet type MMV made of polyacrylamide gel. To validate wet MMVs used for cultivation, bacteria *E. coli* harboring green fluorescent protein (GFP) were subjected to a single cell culture (i.e., a single cell per well (expectation value)). Overnight culture of the cells in the MMV resulted in a Poisson distribution as expected (Fig. 8). This means that we could obtain neatly arrayed colonies without depending on robotics such as Colony Picker [33], which can be easily manipulated in the following processes.

The power of wet MMVs was partly shown above in the success of the PCR-containing experiment (Fig. 4) since the dry MMVs could not have provided a successful PCR due to the drying-up during the PCR thermal cycle. Clearly, the wet gel must have protected the drying-up effect of samples by serving as a reservoir against evaporation. Importantly, whether wet or dry MMVs are used, coating the surface of MMVs with BSA (1 mg/mL) was indispensable for successful reactions.

**Application of MMVs to multistep screening experiments**  
Two independent applications of MMVs were carried out: (a) selection of Amyloid β (Aβ)-binding peptides and (b) selection of cathepsin E inhibitory peptides.

First, the selection of Aβ-binding peptides was performed, consisting of 5 consecutive steps of reaction: PCR-amplification of DNA, *in vitro* transcription, *in vitro* translation, binding of Aβ and Aβ-binding peptides, and monitoring the fluorescence of GFP (which is expressed as a protein fused with Aβ-binding peptides, thus serving as a marker) with three exchanges of MMV plates. The final result of these reactions is the Aβ-binding peptides as shown in Fig. 9 where those wells which contain GFP-fused peptides trapped by Aβ on a magnetic bead are illuminating (dark wells). Those wells where GFPs are expressed and halted (Aβ-binding peptides with a sufficient binding affinity with Aβ can be expected there) indicate the success of a series of reactions. The background brightness is caused by the reflection of the excitation beam on the surface of sample solutions. In this experiment, the peptide sequences could be obtained from the corresponding DNAs contained in the same well by PCR (data obtained are shown in Table 1).

As another example of multistep reactions, selection of cathepsin E inhibitory peptides was performed using MMV plates. For the multistep experiment, wet MMV plates were used for DNA amplification by PCR (starting with 50 molecules of template DNAs [34]), *in vitro* transcription/translation, restriction protease (Xa) digestion, and cathepsin E-inhibition assay (The MMV plate plan used is schematically shown in Fig. 10a). After all, the wells containing cathepsin E inhibitory peptides were marked as a dark spot (Fig. 10b). The contents in those wells were further analyzed by DNA sequencing and the CE-inhibitory peptides were identified (see Additional file 2). Through this experiment, MMVs were again confirmed to be usable for multistep reactions including PCR.

**Conclusions**  
We have first introduced a novel concept microarray that can handle sub-μL scale bulk reactions, which enables 1000-fold parallel and multistep reactions. For this purpose we fabricated wet and dry microarrays with manageable volumes (MMVs). MMVs could be used for setting multi-different conditions and for culturing cells in an array mode (Fig. 6, Fig. 7 and Fig. 8). Especially, MMVs were powerful for *in vitro* selection experiments as shown regarding the selections of Aβ-binding peptides and CE inhibitory ones (Fig. 9 and Fig. 10). Conclusively, the MMV has two prominent merits: an ability to generate multiple conditions (1000-fold and more) and another ability to succeed well-specific products well-to-well (leading to multistep parallel reactions). Both were proven effective experimentally in this paper. We sometimes experienced mis-transfers in the aperture-to-aperture (well-to-well) mode because of side diffusion in-between two MMVs (partly discussed in Fig. 4). This problem must be solved controlling the quality of MMVs (Recently, this was verified by an
experiment using MMVs made of PDMS (data not
shown)). This technology is relatively free from the
problems of evaporation (see Additional file 3) and adhe-
sion losses owing to the direct transfer from well to well
and the introduction of wet vessels made of gel. Since
the volume of a solution is measured by the capacity of
a well, there are some errors in the volume precision by
around 10% or so, which is still permissible for the cur-
rent studies. Moreover, this fault must be conquered by
 improving the surface nature of dry MMVs
(which sometimes work as a scale). Therefore, sub-μL
volume, which is almost beyond the pipette operation,
could be first manipulated quantitatively without
depending on pipettes.

Finally, drawing a possible application of the MMV
technology must be helpful to visualize the utility of this
novel one. Namely, we will be able to fabricate a protein
chip more conveniently and effectively than currently
done. The procedures will be: i) make an oligonucleotide-
array based on the conventional microarray technology,
ii) trap cDNAs by hybridization at each cognitive oligo-
nucleotide spot, iii) transfer each DNA parallelly to each
corresponding well of an MMV by electrophoresis or dif-
fusion (from spot to aperture), iv) do successive reactions

| Round/Clone No. | Nucleotide sequence (5’to 3’) | Peptide sequence (10a.a.) |
|----------------|-------------------------------|--------------------------|
| 1/1            | TGC ATT ATT ATT TGG GAA CAC TCC TGC | CIIIWEHSC               |
| 1/2            | TGC ATT TGC ATT CTC TCA ATT GTG TGC | CIMSIIIVC               |
| 1/3            | TGC ATT AAT ACA GCG CCA AGT AAT TGC | CNNTAPSHNC              |
| 2/1            | TGC ATG TGG TGG ATT CCA ATT AAA CGT TGC | CMWWPIKRC               |
| 2/2            | TGC TGG GTA ATT TGG ATT GTG ATT TGC | CWIWVMMC                |
Figure 10 Selection of cathepsin E (CE)-inhibitory peptides. (a) An MMV plate plan adopted for the selection of CE-inhibitory peptides. First, magnetic beads were added to MMV1 and transferred to MMV2. MMV2 was changed with a cell-free transcription/translation solution. Independently, MMV3 was subjected to amplification of DNA (PCR reaction) and the products were transferred to MMV2. After transcription and translation reactions in MMV2, generated proteins were treated with restriction protease Xa, releasing peptides from the protein bound on the magnetic bead. After removal of magnetic beads, the supernatant was transferred to MMV5 which contains cathepsin E and its fluorogenic substrate. After incubation, the fluorescent product was monitored with a fluoroimager. The symbol ‘arrow with a spiral’ means that the preceding MMV is layered upside down as a donor MMV to the following one (recipient MMV). (b) Selection of CE-inhibitory peptides. In this case, dark wells contain CE-inhibitory peptides which block the CE enzyme reaction (generation of fluorescent products) and result in no production of fluorescent products. The arrows in inset indicate the inhibitory peptide-containing wells.
in MMVs including PCR, *in vitro* transcription and *in vitro* translation on beads within a well, v) transfer the resultant peptides/proteins on beads on a chip surface and fix there by a tag sequence or else, completing a protein chip. This approach will drastically reduce the cost and labor to make arbitrarily designed protein chips. The protein chip thus constructed must be used for ‘proteomic diagnosis’ which aims to survey all biomarkers such as cancer, diabetes, neurodegenerative diseases, and others at once and quantitatively. To be encouraging enough, *in vitro* evolution utilizing the MMV itself (partly shown in Fig. 9 and Fig. 10) must be able to find such biomarkers.

**Methods**

Tools and methods to construct the system of microarray-with-manageable-volumes (shortly, MMV) have been developed in this study, including an MMV generator, various types of MMV plates (made of gel or plastics with wells of different size and number), MMV containers for centrifugation and incubation and sample transfer/solution preparation methods.

**MMV-generator and MMV**

MMVs were fabricated using a home-made apparatus, MMV generator which contains DMD (digital multi-mirror device) projector LVP-XD10 (Mitsubishi, Japan) with the optical system modified (Fig. 2a). This apparatus can project any light pattern under the direction of computer and thus can polymerize gel/plastics, of which reaction can be initiated by light irradiation, in an arbitrary shape (Fig. 2b). A typical dry MMV (1024 wells/inch²) is shown with the dimension of a well (Fig. 2c) and how to fabricate an MMV is depicted (Fig. 2d). Both types of MMVs, wet and dry, were developed using acrylamide aqueous solution and acrylate (pentaerythritol tri-acrylate; Aldrich, Germany), respectively. For the generation of polyacrylamide gel (wet MMV), 18% acrylamide solution (acrylamide:bis-acrylamide = 19:1) containing 0.27 mM riboflavin and 70% sucrose, was used. For acrylate resin (dry MMV), pentaerythritol tri-acrylate, containing 0.45% bis(2,4,6-trimethylbenzoil)phenylphosfinoxide IRGACURE®819 (Ciba, Japan) and 0.05% 2-hydroxy-2-nethyl-1-phenyl-propane-1-on DAROCUR®1173 (Ciba, Japan), was used. After a brief light irradiation using mercury-lump VLT-X10P (Osram, Germany), unpolymerized solutions were immediately removed by jet-water (in case of wet MMV) or by spinning off (in case of dry MMV). The wet MMV was further subjected to a buffer-exchanging process to remove riboflavin, acrylamide monomers and sucrose and replace with an appropriate solution. Thus, the plate of a wet MMV was usually equilibrated with a reaction solution without large molecules such as peptides and DNAs. The swelling/shrinking effect of gels during these processes was kept carefully within control. The dry MMV was finally washed in a labware detergent 7-X ES PF (Dainippon Pharm. Co., Japan) with sonication.

**MMV operations**

Due to the great number (~1000) and the smallness (~0.5 μL) of samples, one-by-one transfer of them is neither reasonable nor realistic. Therefore, we adopted the most direct approach for transferring samples from an MMV to an MMV by attaching two MMVs (donor MMV and recipient MMV) face-to-face and then precipitating by spinning (aperture-to-aperture transfer) and found this working. We further developed necessary techniques for handling MMVs: charging, addition, and selective addition (filtering or template method) of samples, measuring up to a volume, mixing, incubation (cultivation or enzymatic reaction), PCR, separation into solid and liquid parts.

*a) Charging/Addition*

Initial charging of sample solution into each well can be performed by spinning of a pool of solution placed on the top surface of MMV, resulting in filling all the wells with the solution and the remaining solution overflowed over the rim (see Fig. 3b). In case of adding another aliquot to the content of a well in an MMV, a transfer from the concerned MMV to an MMV having deeper wells (recipient MMV) was carried out in advance and then an additive transfer from an MMV (donor MMV) was made (see Fig. 3a). Further additions can be done until the recipient MMV is filled up.

(Development of a robotics is effective but not essential for most experiments.)

*b) Selective addition (filtering/template method)*

In those cases which require addition of a solution to particular wells, we can adopt either a filtering layer method or a template-like MMV one. The filtering layer placed between From- and To-MMVs enables the selective transfer from wells for which the filtering layer is open (penetrable). On the other hand, a template MMV can hold samples in those wells which were made open when the MMV was fabricated and thus can transfer samples selectively from those wells (see Fig. 3c).

*c) Measuring up the volume*

This can be done as an application of *addition* process (see above) if the size of the well of a donor-MMV is made unitary (of a fixed volume). N-times of addition make N units of volume input into a recipient-MMV.

*d) Mixing*

Although such a tiny volume as sub-μL is rather favorable for diffusion and thermal conductance, it is very unfavorable for mixing solutions with a vortex. For this purpose, we made a target molecule (peptide) bound on
a magnetic bead to lift up and drop down by magnetic force. In other cases, stirring the solution with vigorous moving of small steel balls (~0.4 mm in diameter; Super micro-ball generously provided by Toyo Seiko Co., Japan) by magnetic force was also applied, of which effect was confirmed by diffusion of dye.

e) Incubation (cultivation/enzymatic reaction)/PCR
In case of culturing of bacteria, an MMV plate was equilibrated by soaking with LB broth or Davis medium prior to the culture and a container with a large space (more than 18 cm³) equipped with a water reservoir was selected. For a PCR reaction, the MMV plate was placed in a small container (5.4 cm³) firmly sealed to avoid an evaporation loss during incubation and held in a chamber thermally controlled (Mastercycler gradient, Eppendorf Co., Germany). PCR was carried out in a special device for the sake of effective heat conductance and minimum evaporation of water (see Additional file 1). The PCR program adopted was as follows: pre-heating, 94°C, 2 min; denaturing, 94°C, 0.5 min; annealing, 55°C, 1 min; extension, 72°C, 1 min (35 cycles); post-extension, 72°C, 5 min.

f) Separation into a solid and a liquid parts
By attracting magnetic beads upward out of the liquid with a magnet, the solid (beads) and the liquid were easily separated (see Fig. 3d).

g) Image processing (fluorescence/optical light)
A newly made MMV was treated to equilibrate with a permeation solution (50 mM sodium acetate, 0.1 M NaCl, pH 4.5) containing 5 μM fluorogenic substrate of cathepsin E (CE) MOCac-Gly-Lys-DnP-D-Arg-NH₂ (Code 3200-V, Peptide Institute, Inc., Osaka), and was half-filled with a CE substrate and 5 pmol CE additively). This reaction mixture was transferred to a translation MMV by centrifugation. The DNA amplification with this system was confirmed by gel-electrophoresis and silver staining (see Additional file 1).

Polymerase Chain Reaction (PCR) in MMV
A PCR mixture was prepared as recommended by manufacture’s instruction (SpeedSTAR HS DNA polymerase TaKaRa, Kyoto), added optionally with an enzyme stabilizer (Lipidure BL-802 (NOF Corp. Tokyo)), and input into wells of a wet-type MMV which had been equilibrated in advance with PCR buffer containing PCR components except template DNA, Taq polymerase, and primers. The MMV was placed within a PCR container which was hand-made from silicon rubber and stainless-steel sheets (see Additional file 1). The container was placed in a thermal cycler and PCR amplification was performed with a program (pre-denaturation, 94°C, 2 min; denaturation, 94°C, 1 min; annealing, 55°C, 1 min; extension, 72°C, 1 min (35 cycles); and post-extension, 72°C, 5 min). The MMV plate was removed from the container and the content was recovered after centrifugation. The DNA amplification with this system was confirmed by gel-electrophoresis and silver staining (see Additional file 1).

Verification of the MMV transfer operation
To demonstrate the effectiveness of the well-to-well transfer and successive reactions, we constructed and tried a model experiment. A checker-patterned MMV was filled with a PCR solution containing 2 fmole/μL of DNA coding GFP as templates, respectively. SpeedSTAR polymerase (TaKaRa, Kyoto) was used for a rapid amplification and the PCR program was utilized as follows: pre-denaturation, 94°C, 2 min; denaturation, 94°C, 20 s; annealing and extension, 1 min (25 cycles); and post-extension, 72°C, 2 min. After PCR procedures (see ‘MMV operations’ in Methods), the contents of the MMV were transferred to another MMV which was partly filled with a cell-free transcription/translation solution
T3/T7 are symmetrical). Then, this MMV was incubated at 37°C for 1 h. The fluorescent image was monitored with a fluoroimager and the checkered fluorescent image was obtained. The consistence between the PCR well-pattern and the fluorescent image of GFP verified the fidelity of MMV transfer operations (see Fig. 4).

**2N Method**

In the case of micro-arrays which have a $2^N \times 2^N$ square well-pattern ("N" designates a natural number), its whole diversity ($2^{2N}$) can be generated by employing “2N” kinds of quite different (orthogonal) elements. “2N” kinds of elements can be easily prepared by using “2N” sheets of filters (by considering the symmetrical nature of filters, “N” is sufficient) which have rather simple well patterns. In the case of “N” = 2 (see Fig. 5) for example, 2 sheets of filter are sufficient to be prepared and each filter can be used in two ways, working virtually as four template plates. Four kinds of elements are transferred into a recipient microarray by centrifuge. Finally, all of the 16 wells of the microarray would have different constituents from each other. Each well can be uniquely assigned by the binary number. If we regard the digit 1 as ‘exist’ and 0 as ‘non-exist’ and if we consider the difference of the order in the number corresponds to the difference of elements in the actual experiment, then the well assigned 1001 (9 in the decimal number) should contain the elements corresponding to the elements #1 and #4 (#1-#4 corresponds to the MSB, 2nd MSB, 3rd MSB and LSB). Here, we utilized a 256-well micro-array ("N" = 4) for examining condition on re-crystallization of lysozyme (see Fig. 2f where we can confirm that the pairs of T0/T4, T1/T5, T2/T6, and T3/T7 are transferred into a recipient microarray by centrifuge.

**Library construction and selections**

**a) Library construction**

Combinatorial DNA and IVV (in vitro virus)-peptide libraries were constructed according to the previously reported method of YLBS [35] and cDNA display [36].

**b) Preparation of CE-immobilized beads used for selection**

Purified cathepsin-E (CE) was immobilized on NHS-activated sepharose beads (GE Healthcare, USA) by using the amine coupling chemicals to form a chemically stable amide bond in accordance with the manufacturer’s instructions. The enzyme-coupled beads were stored in 100 mM phosphate buffer (pH 7.4) at 4°C until further use. The coupling efficiency was calculated by comparing the absorbance of uncoupled enzyme with that of free enzyme.

**c) Affinity-based selection**

The IVV-peptide library in 100 μL of Selection buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, pH 7.2) was mixed with 5 μL of CE-immobilized beads and incubated at 25°C for 30 min. The beads were washed with 200 μL of Selection buffer, Washing buffer-1 (50 mM Tris-HCl, 0.5 M NaCl, pH 7.2) and Washing buffer-2 (50 mM Tris-HCl, 1 M NaCl, pH 7.2) in accordance with the protocol of washing repeat count, which varied along the selection round, i.e., 2:2:1 in Round 1; 2:2:2 in Round 2; 3:3:3 in Round 3. Finally, the beads were suspended in 200 μL of Elution buffer-1 (50 mM Tris-HCl, 1 M NaCl, 10 mM MgCl2, pH 7.2) and incubated at 37°C for 5 min. After centrifugation (1500 rpm, 1 min), the beads were washed again with Elution buffer-1. The combined supernatant was stored (Sup1). The beads were suspended with Elution buffer-2 (50 mM Tris-HCl, 2 M NaCl, 10 mM MgCl2, pH 7.2) and incubated at 95°C for 5 min. After incubation, the beads were removed and the supernatant was stored (Sup 2). The IVVs in Sup1 and Sup 2 were purified with a BioSpin column (Bio-Rad Laboratories, USA).
**d) Function-based selection**

The DNA library resulting from Round 3 of the affinity-based selection was inserted in the IVV-SF-link DNA construct as a variable region [36]. The IVVs were prepared and incubated as described in affinity-based selection. The protocol of washing repeat count with Selection buffer, Washing buffer-1 and Washing buffer-2 was carried out according to the selection round program (F1 to F9) as follows: 3:3:3 in F1, 4:5:3 in F2, 5:5:5 in F3, 5:7:5 in F4, 5:7:7 in F5, 5:10:7 in F6, 5:10:10 in F7, 5:15:10 in F8, and 5:15:15 in F9.

**Sample preparation**

**a) GFP-expressing Escherichia coli**

The buffer components in a gel-MMV plate (1 × SSC) were exchanged with LB broth and the affluent solution in wells was removed by centrifugation. Overnight culture of *E. coli* cells harbouring green fluorescence protein (GFP) was diluted with LB broth to the concentration of one cell per well (0.5 μL) and then put into wells of an MMV by centrifugation. The MMV was layered on a wet tissue paper in a petri dish and subjected to the incubation at 37°C. The fluorescence of GFP expressed in *E. coli* cells was monitored with a fluoroimager, Molecular Imager FX (Bio-Rad Laboratories, USA).

**b) A DNA library for cathepsin E-inhibitory peptides**

A DNA library was constructed, of which DNA encodes a different species of a cathepsin E-binding peptide (8-28 amino acids in size) which is derived from the preceding in vitro selection experiment [36] (see ‘Library construction and selection’ and Fig. 11).

**c) Lysozyme solutions**

Lysozyme provided in lyophilized powder (protein content, ~95%; ~50,000 units/mg protein (Sigma-Aldrich, Germany)) was dissolved in distilled water (250 mg/mL) using a vortex mixer with a special care so as not to leave visible flocs and then spun down. The supernatant was used for the crystallization experiment.

**Generation of combinatorial conditions (2N method)**

We applied the 2N method (see ‘2N Method’ and Fig. 5) using a 256-well MMV to generating 256 species of conditions aimed for crystallization of lysozyme. The ionic strength and pH were modulated using the following NaCl and sodium acetate-HCl buffers, respectively. Each template MMV of T0~T7 was charged with 0.1 μL of 2 M NaCl (T0), 3 M NaCl (T1), 4 M NaCl (T2), 5 M NaCl (T3), pH 3 (T4), pH 4 (T5), pH 5 (T6), and pH 7 (T7), respectively. To make the final solution volume equal in each well, the complementary volume of water was added to those wells which were not charged with any of T0~T7 solutions, using the complementary templates T0~T7 (well/non-well relations inverted). Finally, a lysozyme solution (250 mg/mL) was added (0.1 μL). The MMV was covered with a piece of transparent adhesive tape Titer Stick (Wakenyaku, Japan) to prevent evaporation and was kept at 20°C for several days. Each well was monitored by an inverted microscope IM (Olympus, Tokyo).

**Multistep operations of MMVs**

**a) In vitro selection of Aβ-binding peptides**

The whole procedure is described in detail above, in which the construct and the selection of a peptide-fused
GFp is described (see Fig. 9). Peptides were expressed by successive reactions of PCR, in vitro transcription and in vitro translation as written above. Then, Aβ protein (Aβ42) linked to a magnetic bead was added by an MMV-transfer (i.e., transfer from an MMV to another MMV by centrifugation). Those peptides bound to Aβ were separated from the remaining unbound ones by the next MMV-transfer (see ‘MMV operation’), washed with a buffer (10 mM Tris-HCl, 1 mM EDTA, 1 M NaCl, 0.1% NaN3), and then subjected to the fluorescence monitoring using fluoroimager Molecular Imager FX (Bio-Rad Laboratories, USA) at the excitation wavelength of 488 nm.

b) In vitro selection of cathepsin E-inhibitory peptides
A set of peptides which have a cathepsin E-inhibition activity selected in the preceding study [34] were further screened using MMVs (see Fig. 10). The DNA molecules encoding those peptides were diluted and input in an MMV (~50 copies per well) and then subjected to PCR, in vitro transcription/translation, and protease Xa digestion. Then, the magnetic beads were removed from the MMV. The remaining solutions were used to detect the inhibition activity of the peptide in each well. The volume of the reaction mixtures were, if too much, reduced to around a half-well volume by sucking with filter paper. The measurement of the inhibition activity in each well was carried out as described in Methods (Image processing).

Additional file 1: Additional Figure 1 - PCR reaction using an MMV
(a) PCR device specialized for the microarray. Upper cover and Bottom container were fabricated of stainless steel sheet and silicon rubber. The microarray in the container was heat-treated via the heat block of PCR instrument. To avoid the leakage of vapor from the container, Upper cover was pressed with a high temperature lid which prevented the vapor from condensing on the cover. (b) PCR product (780 bp) was recovered by centrifuge and analyzed by polyacrylamide gel electrophoresis and silver staining. DNA templates were amplified from 50 molecules (lane 1) and 10 molecules (lane 2) per well, respectively. Lane "M" shows marker DNA bands (upper: 850 bp, lower: 750 bp).

Additional file 2: Additional Table 1 - Cathepsin E-inhibitory peptides obtained by the MMV method. A list of peptides selected by the MMV method is given.

Additional file 3: Additional Figure 2 - Evaporation rate curve. Evaporation rates were measured with an MMV filled with water placed on ice (square) or on the lab bench (triangle) under the conditions of room temperature (~15°C) and humidity (47% and 58%, respectively).

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Authors’ contributions
YK and TT contributed equally. YK performed most of the finalizing experiments for optimizing and improving MMVs and obtaining the data and jointly wrote and edited this paper. TT carried out the most of the initial and advancing stage of this study. KK and MSa made the application and jointly wrote and edited this paper. TT directed the experiment and jointly edited the paper. YH efforted to begin and promote this study and provided essential discussions. KN designed, designed and wrote and edited this paper. All authors read and approved the final manuscript.

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