Supplementary Information

Liquid-capped encoded microcapsule for multiplex assays

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Materials and Methods

Section S1: Fabrication of liquid-capped microcapsule

Fabrication of microfluidic devices

Polydimethylsiloxane elastomer (PDMS, silgard 184, Dow Corning) was poured onto a silicon wafer that is patterned with SU-8 photoresist (SU-8 2015, SU-8 2050, MicroChem) using photolithography and thermally cured for 15 min on a 150°C hotplate. After curing, the PDMS elastomers were peeled off from the silicon wafer. The PDMS channel surface was made hydrophilic as follows. First, the two slabs of the PDMS channel with a hillock were prepared. Holes for the inlets and outlets were created, and the slabs were washed with ethanol and dried with nitrogen gas (N2). Then, the PDMS slabs were treated with oxygen plasma for 1 minute (CUT-MP, Femto Science). Before the hydroxyl groups of the PDMS surface disappeared, two slabs were attached to form one united channel with a height of 200 μm under a microscope. The bond between the two slabs was strong enough to allow the flow of liquids inside the channel. The bonded slabs were attached to a glass slide. Within 5 minutes, the silane coupling agent, 2-[methoxy(polyethyleneoxy)propyl]trimethoxysilane (Gelest) in toluene (at a volume ratio of 9:1) was introduced into the assembled channel using a suction equipment for 20 seconds. The solution residues and bubbles cause non-uniform surface modification. When the reaction solution was introduced into the channel with the suction equipment, the residues and bubbles were effectively removed. Moreover, the suction equipment allowed selective surface modification of the PDMS channel because the strong suction prevented liquid overflow from the outside into the hillock side. The channel was then left at room temperature for 5 minutes under pressure. The toluene was poured into the channel for several seconds, and the channel was washed with ethanol for 30 seconds. Channel bonding was maintained during the flowing process of these several liquids. Finally, the channel was heated on a hotplate at 100 °C for 10 minutes (Fig. S1).

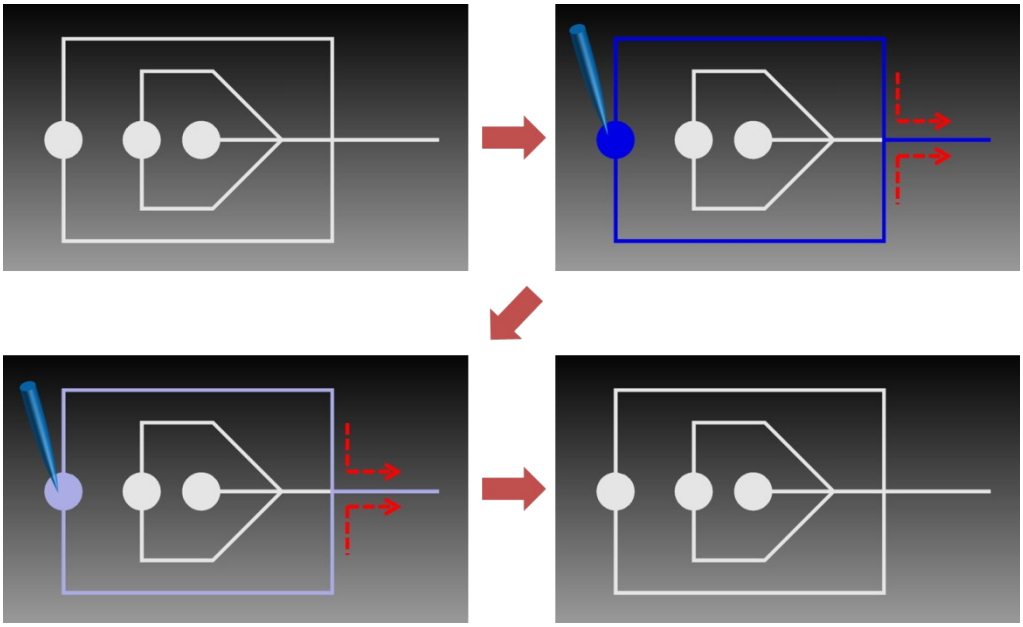

Fig. S1. The schematic of PDMS surface modification. After plasma treatment and bonding of two PDMS channel, the channel is treated with the silane coupling agent and washed with toluene and ethanol sequentially.

Generation of liquid capped microcapsule

To make liquid capped microcapsules, we used microfluidic device generating double emulsion droplet by 3D coaxial flow with simple PDMS hillock structure[1]. By selectively hydrophilic coating, stable water/perfluoropolyether (Fluorink® PFPE MD700, Solvay Solexis Korea)/water with 2% surfactant (BASF) double emulsion droplets are generated. The hillock structure allows outside PFPE with 3wt% photoinitiator (2, 2-dimethoxy-2-phenyl-acetophenone, DMPA, Sigma Aldrich) liquid to enclose the core liquid and prevent the core flow attaching the channel surface. After the core liquid droplets are generated by PFPE/initiator mixture, outer water breaks the core-containing PFPE phase, which generates double emulsion droplets. The PFPE polymer is polymerized by UV irradiation (wavelength: 365 nm, 50mW/cm^2) for 10 seconds and the core liquid is encapsulated by a polymerized PFPE microcapsule. In previous work of Vitale and coworkers[2], polymerization kinetics of PFPE was studied by three different techniques, FT-IR analysis, DSC analysis and insoluble fraction measurement. They proved that the conversion of polymerization reaches 100% at about 60 seconds of UV irradiation at 3mW/cm^2. In our experiment, the FT-IR analysis of polymerized PFPE according to various UV exposure time shows that the PFPE is fully polymerized more than 25s of UV exposure time at 15mW/cm^2 (Fig. S2).

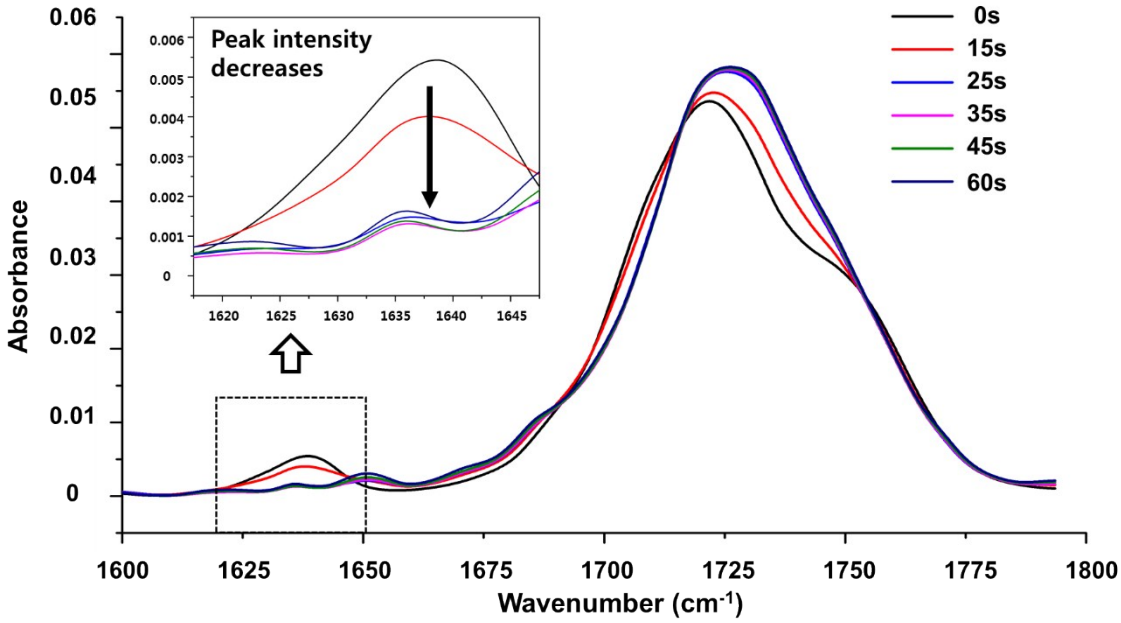

Fig. S2. FT-IR analysis of polymerized PFPE according to various UV exposure time. PFPF with 3wt% of DMPA is polymerized at different exposure time (from 0 to 60s) at at 15mW/cm^2. Conversion of PFPE monomer to polymer is observed by monitoring methacrylate C=C peak at 1632 cm^{-1}. PFPE is fully polymerized more than 25s of UV exposure time.

Flow rate control for generation of double emulsion

In order to find suitable flow rate condition for generation of microcapsule, we varied either core or outer phase flow rate by fixing the other flow rates. In low outer flow rate, double-core emulsions are generated because outer flow rate is not sufficient to break the middle phase to have only one core phase droplet. In high outer flow rate, satellite drops are generated because outer phase break the middle phase before entire core phase droplet pass the junction of the channel (Fig. S3A). In low core flow rate, it leads to multiple drops in PFPE droplet. As we increase the core flow rate, diameter of core drop is increased and thickness shell is decreased. Here, size of double emulsion droplet is not significantly increased because the size of droplet is mainly affected by dimension of microfluidic device. At high core flow rate, the thickness of shell is too thin and core liquid is burst (Fig. S3B).

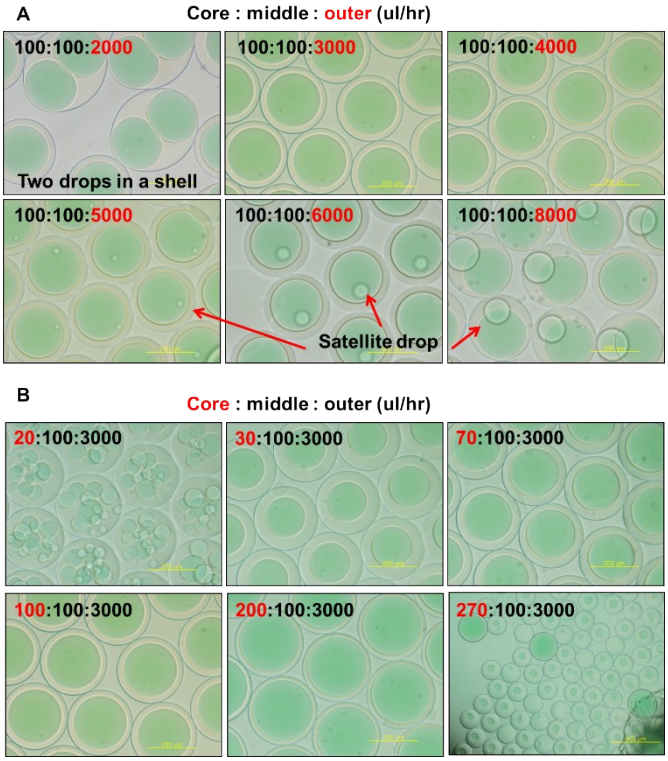

Fig. S3. Flow rate control results. (A) Outer flow rate control results. The flow rates of the other phases are fixed. Two insets show figures of junction in microfluidic channel in each flow rates. (B) Inner flow rate control results.

Section S2: Encoding of the microcapsule

Encoding process

The polymerized PFPE microcapsules are packed on a transparent substrate to consist monolayer of the microcapsules and patterned UV light (wavelength: 365 nm, 180 mW/cm^2) is illuminated on the shell of microcapsule. There are two encoding methods. One is using digital micromirror device (DMD, Texas Instrument) and another one is using patterned physical mask. DMD acts as a computer-controlled spatial light modulator and by changing the pattern of the DMD, patterned UV light is illuminated on the microcapsules and various codes can be simply encoded without physical mask (Fig. 2E). As another method, the film-combined glass mask (FCG mask, Microtech, South Korea) was used to generate patterned UV light. The patterned film was attached to the glass. The microcapsules were placed on the FCG mask and UV light was illuminated from the bottom of the mask. When the UV light passed through the mask, the opaque parts of the mask blocked the UV light. Thus, the only patterned UV light reached the microcapsules. Considering the illuminated region is not limited to the field of view of the microscope, whole microcapsules on the mask can be exposed to UV light

and high-quantity encoding of microcapsules is possible (Fig. S4). In both case, the code areas of polymerized microcapsules were selectively exposed to UV light for 60 seconds and encoded microcapsules were fabricated. The code on the microcapsule did not disappear even in additional UV light exposure to the entire area (Fig. S5).

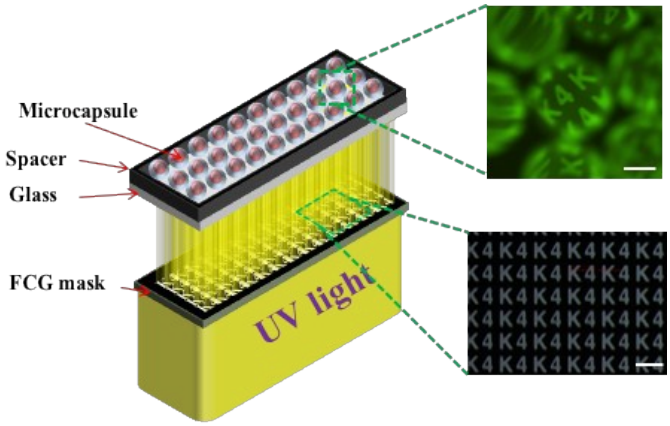

Fig. S4. Schematic of encoding process. Encoding methods using patterned physical mask.

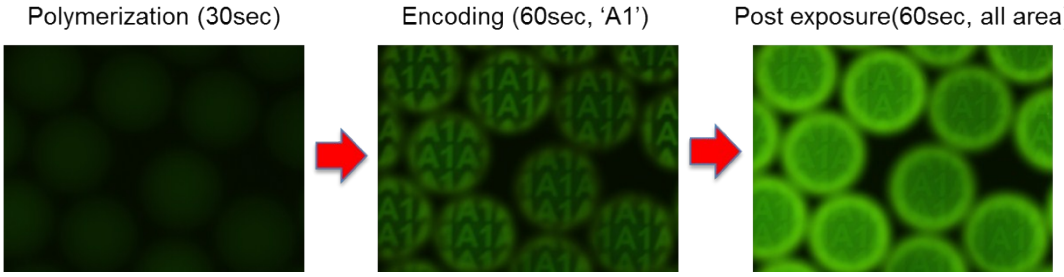

Fig. S5. Two-step UV light exposure process for encoded microcapsule. To generate encoded microcapsule, microcapsules are first exposed to UV light for polymerization (30sec) and patterned UV for encoding (60sec). The code on the microcapsule exists even in additional UV exposure to entire area (60sec).

Variation of fluorescence intensity according to UV irradiation

The fluorescent intensities of the code also depend on the initiator concentration in the PFPE (Fig. S6). The PFPE solutions with three different concentrations of DMPA photoinitiator were prepared 1 wt%, 3 wt%, and 10 wt%, respectevely. These solutions were poured onto glass slides and exposed to the patterned UV light for 30 seconds at 180 mW/cm^2. The fluorescent images were captured and the pixel color information was sampled. The intensity values were obtained and plotted on the graph. Given that the DMPA photoinitiator itself has photoluminescence under UV light, the intensity increased as the concentration of photoinitiator increased.

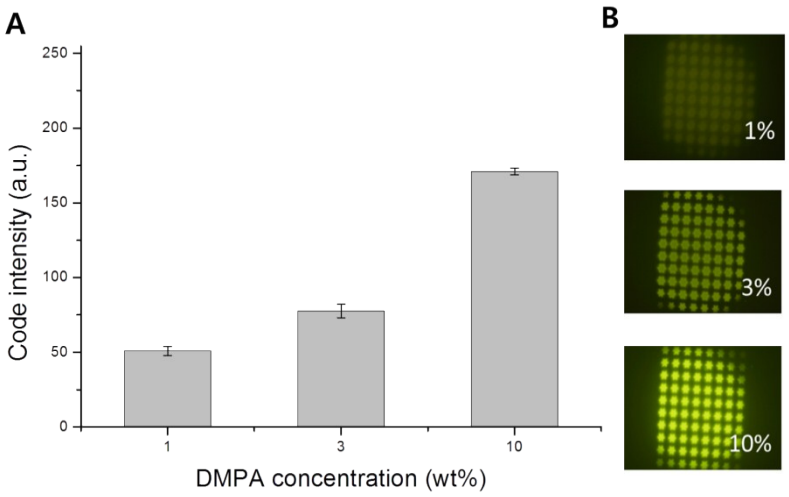

Fig. S6. Fluorescent intensity variation with the different concentrations of DMPA photoinitiator. (A) Releationship between DMPA concentration and code intensity. (B) Code intensity difference according to the variation of DMPA concentration. Standard deviation was multiplied by three.

Durability of code on the microcapsule

To verify long time persistence of the code of microcapsule, microcapsules were prepared and encoded to test the durability of code. Firstly, microcapsules were dispensed in a 96-well plate. The patterned UV light (365 nm, 180 mW/cm^2) was illuminated to the capsules through an objective lens using DMD device. Different characters on the shells indicate different UV irradiation times (character L: 10 seconds, N: 15 seconds, I: 20 seconds, B: 30 seconds). All cases exhibited long term code durability (Fig. S7A). No significant decrease in code intensity was observed in these cases. Thus, UV illumination needs at least 10 seconds at 180mW/cm^2 to generate codes. Among the encoded microcapsules, code character B was continuously monitored for 58 days and the intensity did not decrease significantly for 58 days (Fig. S7B).

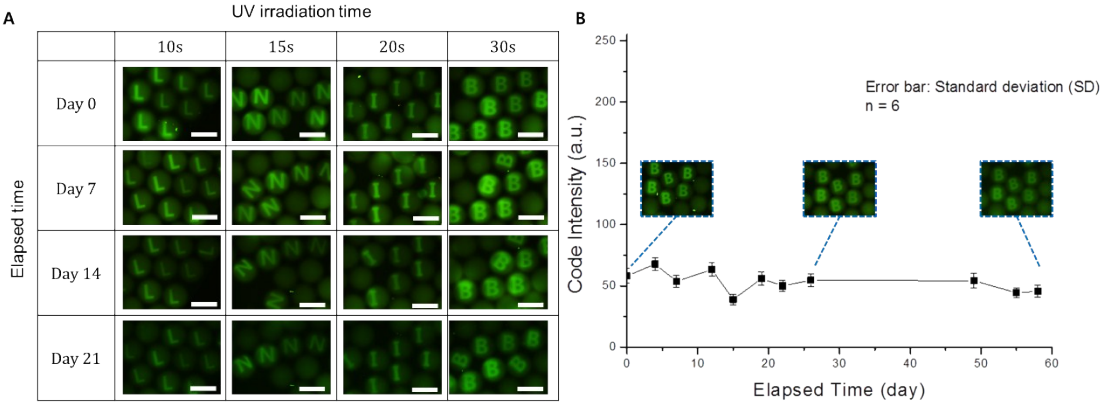

Fig. S7. Code durability test. (A) Code durability with the different UV irradiation time. (B)

The graph for the code intensity of “B” encoded microcapsules.

Section S3: Microwell fabrication

Fabrication of microwell and micropillar

PDMS microwell for laser releasing system was fabricated by conventional soft lithography technology. For polyurethane acrylate (PUA) microwell generation for mechanical releasing system, a UV curable PUA monomer (MINS-311, Minuta Co., Korea) was poured on a PDMS master mold and an adhesion primer (Minuta Co., Korea) coated slide glass was placed on top of the uncured PUA monomer layer. After pushing the slide glass to make flat PUA surface, The PUA layer is exposed to UV (λ = 365 nM, 110mW/cm^2) for 60 seconds. The cured PUA microwell on the slide glass is detached from PDMS master mold and the detached PUA microwell is additionally cured for 60 seconds. Then, the slide glass is washed with ethanol to remove remaining adhesion primer on the surface of glass, which can be toxic to the cell. In the case of micropillar array, flexible PET film (thickness = 100μm) was placed on top of the uncured PUA which was poured on the PDMS mold and exposed to the UV light. As the PET film has good bonding property with PUA material, it does not need to coat the surface of PET film with adhesion promoter. After UV curing, the cured micropillar array is peeled off from the PDMS master mold (Fig. S8).

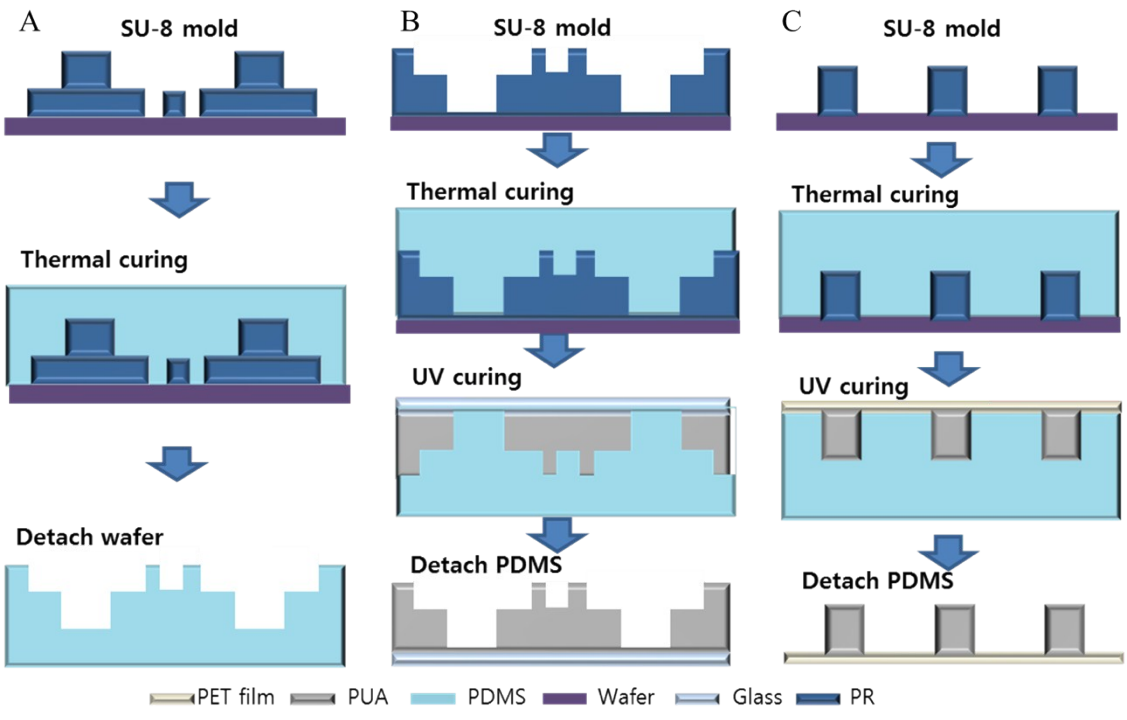

Fig. S8. Fabrication process of microwell and micropillar array. (A) PDMS microwell fabrication. the PDMS microwell is fabricated by conventional soft lithography process. (B) PUA microwell generation process. The SU-8 microwell mold is double-casted with PUA monomer onto the adhesion promoter coated glass slide. (C) micropillar array generation. SU-8 micropillar array mold is double-casted with PUA monomer onto the flexible PET film.

Designing of the microwell

The microwell can consist of one or two layer. In the case of one layer microwell, height of microwell (h1) should be higher than diameter of a microcapsule (d) and smaller than one and a half diameter of a microcapsule, which enables extra microcapsules to be removed easily by sweeping after assembly of microcapsules. In the case of two layer microwell, height of the first (bottom) layer is same with the height of one layer microwell and height of the second layer should be smaller than a half of the diameter of a microcapsule for the same reason (Fig. S9A, B). Also, by changing design of microwell, various combinations of the encoded microcapsules can be achieved (Fig. S10).

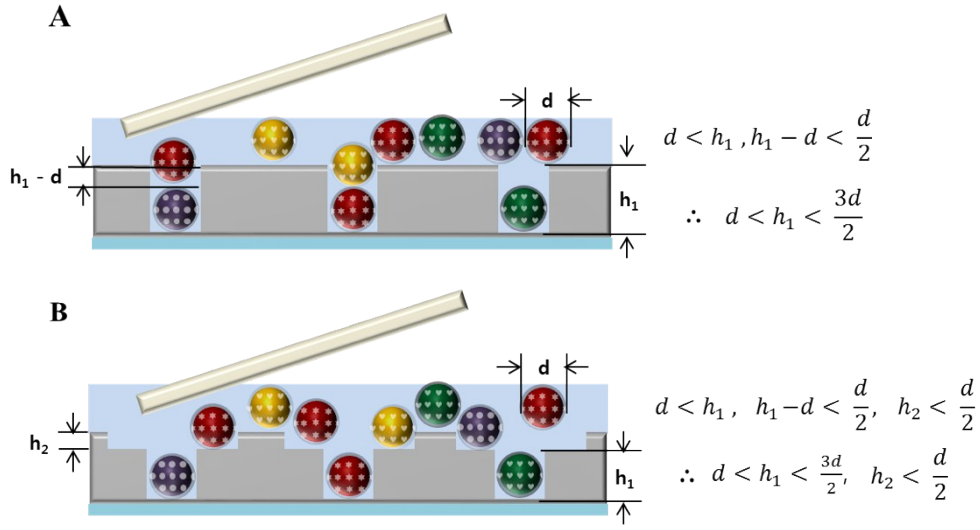

Fig. S9. Desings of microwell array. (A) Dimensions of one layer microwell. (B) Dimensions of two layer microwell.

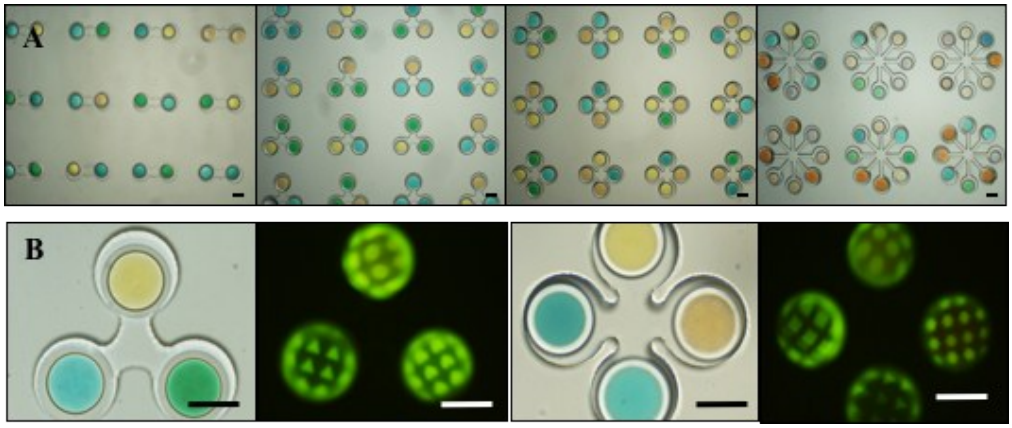

Fig. S10 Combinatorial assembly of the differently encoded microcapsules. (A) Various designs of microwell combinations. (B) The combination of liquids can be identified by decoding the code of the microcapsules in microwell.

Section S4: Assembly of the encoded microcapsule

Binomial distribution of the encoded microcapsules assembled in microwells

To estimate the code distribution of the encoded microcapsules after assembly, we assumed that the assembly of the microcapsules with 10 different codes. When a microwell is filled with a microcapsule, the probability of a microcapsule with specific code is p = 1/10, while the probability of microcapsules with other codes is 1-p = 9/10. When we consider N microwells where the microcapsules are assembled, the probability of microcapsule with a specific code in k microwells follows a binomial distribution. A random variable X follows a binomial distribution with parameters n, k, and p, where, n is number of trials, k is number of successes, and p is probability in each trial. The probability mass function of X follows equation.

$$f(k;n,p) = \Pr(X = k) = \binom{n}{k} p^k (1-p)^{n-k}$$

Fig. S11. show that the probability density function of binomial distribution for the assembly of the encoded microcapsule with a specific code. Here, we assumed that the microcapsules have 10 different codes and are assembled in 100 microwells. When we consider minimum value of the number of assembled microcapsule from our experiment, the probability that more than 4 microcapsules with a certain code are assembled in microwell is about 99.22%.

$\Pr(4 \leq X) = 0.9922$

Theoretically, the average number of microcapsules with a certain code in 100 microwells is E(X) = np = 10. If we increase the number of the microwells, all drug candidates can be covered at one experiment with high probability.

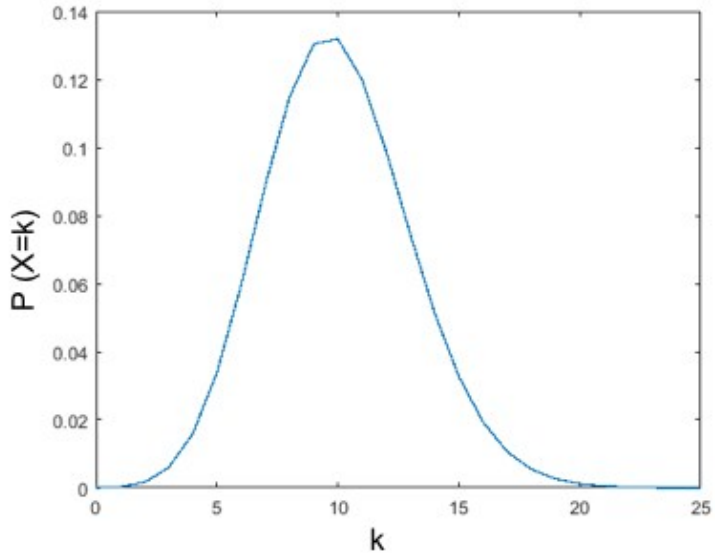

Fig. S11. Probability density function (X=*k*) generated by a binomial distribution for the assembly of the encoded microcapsule with a specific code. Assumed that microcapsules with 10 different codes are assembled in 100 microwells.

Section S5: Releasing of the liquid inside the microcapsule.

Laser releasing system

For reaction of our platform, the core liquid should be released from the shell of the microcapsule and be reacted with surrounding liquid in the microwell. Here, we developed pulse laser equipment utilizing laser induced ablation of microcapsule (Fig. S12). The equipment consists of motorized moving stage, CCD camera and nanosecond pulse laser (Minilite Nd:YAG laser, Continuum Inc., maximum power: 12mJ at 532nm, pulse duration: 7ns). The microcapsules are assembled in microwell and the microwell is sealed with slide glass to prevent evaporation of surrounding liquid in microwell and placed on the motorized stage. By applying on laser pulse, the shell of the microcapsule is broken by laser ablation and core liquid inside the microcapsule is released to the microwell and mixed with surrounding liquid. This laser breakage system enables selective and sequential releasing of core liquid in microwell array.

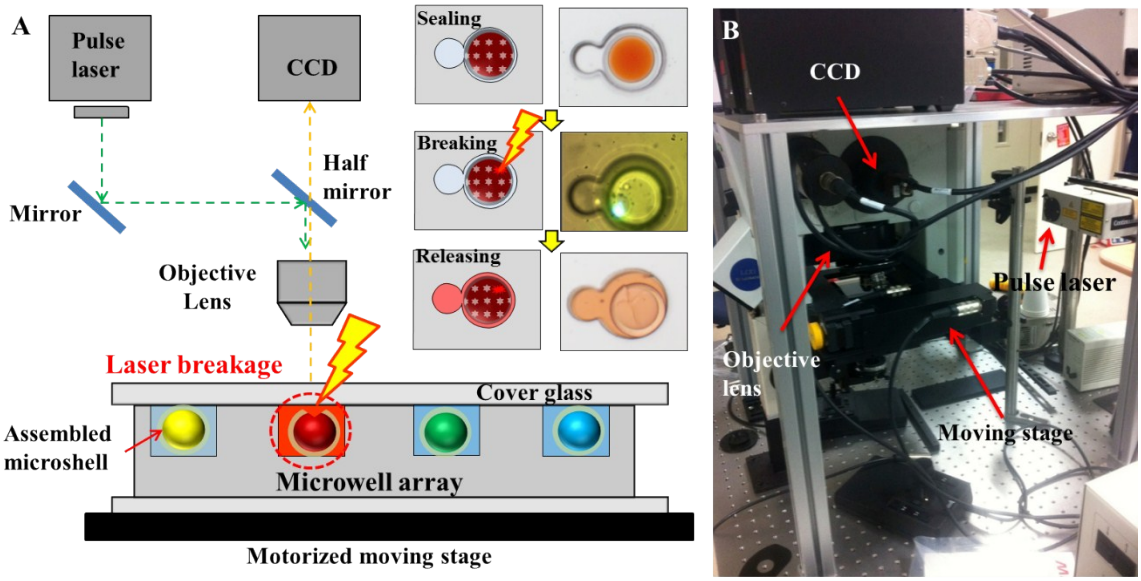

Fig. S12. Pulse laser releasing system. (A) Schematic image of pulse laser releasing process. After assembly of microcapsules, microwell array is sealed by slide glass to isolate each well and prevent evaporation of surrounding medium. When the pulse laser is focused and applied on shell of the microcapsule, the shell is broken by laser ablation and core liquid of microcapsule is released and diluted with surrounding liquid of microwell. (B) Experimental setup of pulse laser releasing system. The setup consists of Nd:YAG pulse laser, CCD and motorized moving stage.

Mechanical releasing system

In addition to laser releasing system, we also developed mechanical releasing system utilizing micropillar array (Fig. S13). The plaform where the microcapsules are assembled in the polyurethane acrylate (PUA) microwell is first covered with the immiscible silicone oil to prevent reagents evaporation and cross-contamination between microwells. The micropillar array is then aligned with the microwell array and smoothly pushed and swept with to break the shell of the microwell. After breakage process, various types of core liquids in the microcapsules are released into the individual microwell and react with regent filled in the microwell.

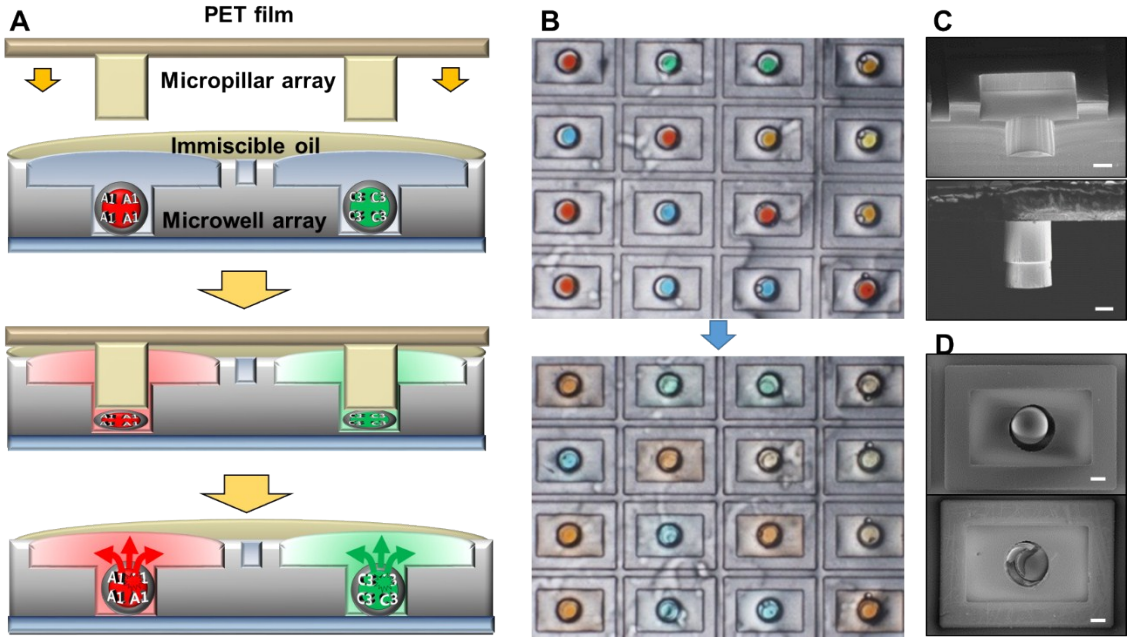

Fig. S13. Mechanical releasing system. (A) Schematic images of mechanical releasing system. After assembly of microcapsules, the microwell array is sealed with immiscible oil to prevent evaporation and cross contaimination. Micropillar array fabricated on flexible PET film is then aligned with microwell array. By pushing micropillar array, the microcapsules are burst and the core liquid is released. (B) Images of microwell array before and after releasing. The colored core liquid is released and diluted with liquid in the microwell. (C) SEM images of the microwell, micropillar and microcapsule assembled in microwell before and after breakage.

Automated mechanical releasing equipment

For automation of mechanical releasing method, we employed motorized stage and ball bearing equipment (Fig. S14). The microwell and micropillar array is aligned on the motorized stage and the ball bearing equipment is aligned at the microwell to be scanned. After pushing and fixing z-axis of the ball bearing on the micropillar array substrate, the motorized stage moves toward designed route to scan whole microwells. As micropillar which is forced by ball bearing squeeze each microcapsule, the microcapsule is ruptured and core liquid is released.

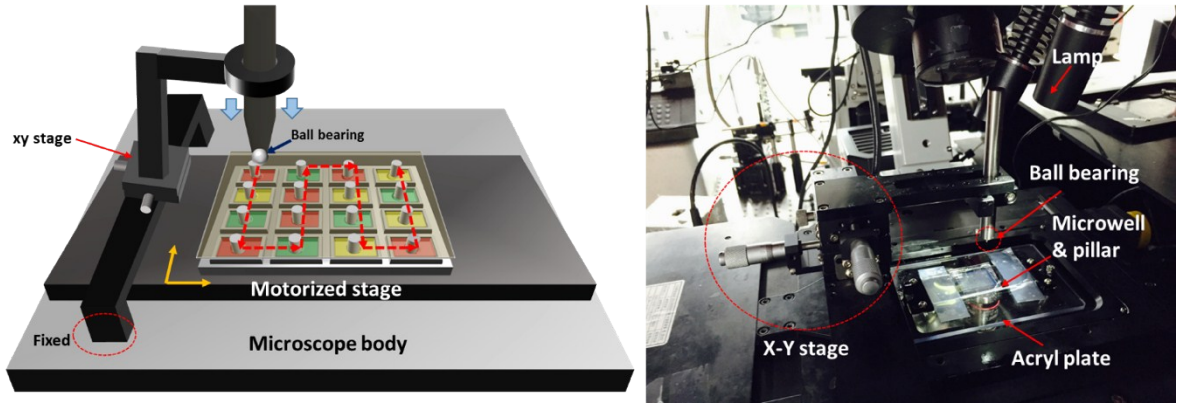

Fig. S14. Automated mechanical releasing equipment. The equipment consists of motorized stage on the microscope, ball bearing and x-y stage to control location of ball bearing.

Section S6: Properties of the encoded microcapsule

Leakage test

For generation of a pooled microcapsule library, it is necessary that the core liquid should be stably sustained inside the microcapsule. To verify stability of encapsulation, microcapsules containing fluorescent dye (Fluoroscein, Sigma-Aldrich) was fabricated and assembled in microwell array. After assembly, the microwell was sealed by slide glass and fluorescent intensity of surrounding liquid in microwell was measured for a month. For a comparison, microcapsule in one microwell was broken and the fluorescent intensity of released fluorescent dye was measured (red dot line). The measured fluorescent intensities are shown in Fig. S15. The graph shows that there is almost no leakage from microcapsule and the core liquid is stably encapsulated by polymerized PFPE shell for a long time.

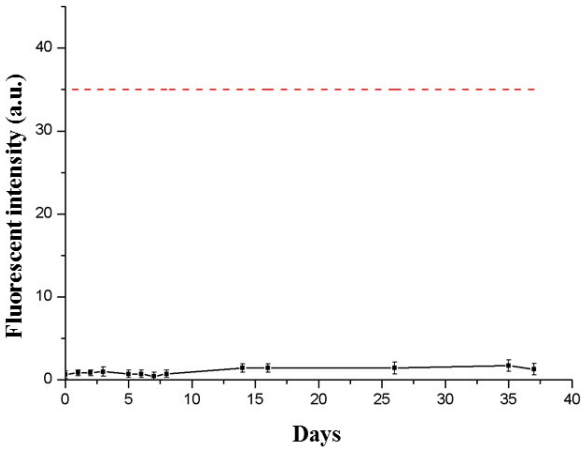

Fig. S15. Results of leakage test. Fluorescent intensity of surrounding liquid in microwell. Microcapsule containing fluorescent dye (fluorescein) was assembled in each microwell and

the fluorescent intensity was measured for a month. Red dot line represents reference intensity when the microcapsule was broken and the fluorescent dye in microcapsule was released in microwell.

Measurement of off-centered degree

To verify effect of inhomogeneous shell of microcapsule to the assembly, we observed cross section view of assembled microcapsule. For this, we exchanged stock solution of microcapsules to photocurable PEG-DA solution. We diluted the PEG-DA with D.I water to decrease viscosity of solution and enable the microcapsules to move freely during assembly process (PEG-DA : D.I water = 1 : 20). After assembly of the microcapsules, the PEG-DA solution is cured by UV light and cured PEG-DA is detached carefully from the microwell array. The microcapsules are trapped in cured PEG-DA layer and by slicing the PEG-DA layer, we observed cross-section view of assembled microcapsules (Fig. S16).

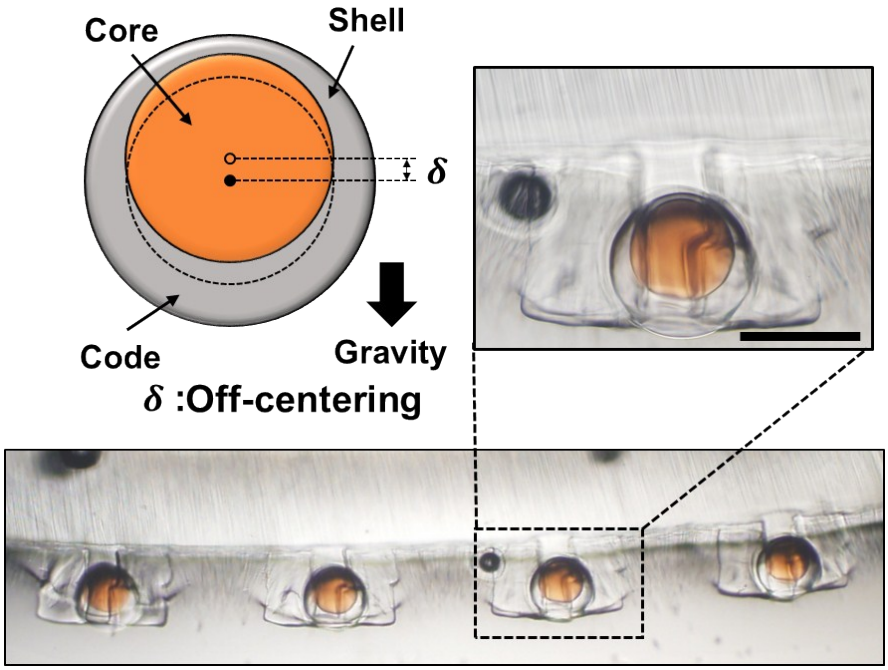

Fig. S16. Off-centering feature of the microcapsule. The images show cross-section view of the microcapsules assembled in the microwells. The microcapsules rotate during assembly process to minimize gravitational potential energy and thicker part of shell faces downward, which has advantage in encoding and decoding process.

Long time storage test of the liquid inside the microcapsule

To verify stability of core liquid inside microcapsule, long time storage test is also conducted to identify whether the property of chemical compound in microcapsule remains

with the passage of time. We performed simple enzyme-substrate reaction with β-galactosidase in microwell and fluorescein-di-beta-D-galactopyranoside (FDG) in microcapsule. When the microcapsule is broken and the FDG substrate in microcapsule is released into microwell, The FDG is sequentially hydrolyzed by β-galactosidase to highly fluorescent fluorescein (Excitation/Emission = 492/520 nm) and the fluorescent intensity was measured according to reaction time (Fig. S17). The graph shows that the chemical inside microcapsule can maintain its property until 20 weeks.

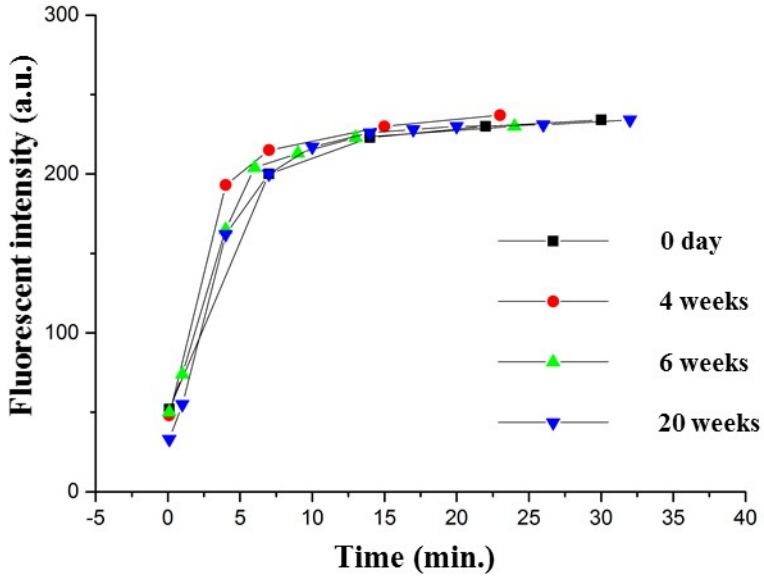

Fig. S17. **Long time storage test according to passage of time.** The graph shows that time-course fluorescent intensity of enzyme-substrate reaction according to various storage periods.

Section S7: Drug-induced apoptosis test

Cell culture on the microwell array

U2OS human osteosarcoma cells were cultured in McCoy's 5A culture medium with 1% of penicillin-streptomycin and 10% of Fetal Bovine Serum at 37°C under 5% CO2 incubation chamber. Cultured cells were detached from culture flasks using 0.25% trypsin and 0.13% EDPT in phosphate buffered saline (PBS). The centrifuged cells were dispersed in a cell culture medium prior to seed. The PUA microwell array was sterilized in an autoclave for 20min under 120°C, followed by oxygen plasma for 1 min. Then the PUA microwell array was treated with 5μg/mL fibronectin solution in PBS, incubated for 1h and then washed with PBS three times. Cells were seeded in PUA microwell array and allowed to settle down into microwell array for

30 min. The seeding density of cells was adjusted to set a cell density of approximately $4x10^5$ cells/mL, resulting in about 200 cells per well. After cell seeding, cells in the microwell array were cultured in incubation chamber for 12h prior to use.

Process of drug-induced apoptosis test

Stock solution of microcapsules containing anticancer drug were exchanged with serum free medium prior to use and the PUA microwell array is immersed in serum free medium. Microcapsules were assembled into microwell array by sweeping several times and the microwell was sealed with silicone oil. The PUA microwell array was then aligned with PUA micropillar array and microcapsules were broken by mechanical force and core drugs were released into the each microwell. After releasing of drug, the PUA microwell array was incubated for 12h to allow reaction between drugs and cells. After reaction with the drugs, the PUA microwell array was gently immersed into PBS to wash and remove silicone oil which covered chip and stained with apoptosis detection solution (FITC annexin V apoptosis detection kit, BD PharmingenTM) and membrane permeable DNA staining dye (Hoechst 33422, Invitrogen) for 1h in an incubator.

Section S8: Image processing

Image processing for code recognition

After assembly of the encoded microcapsules, images of whole microwell were obtained. Then, the code of each microcapsule in each position of the microwell are recognized and matched with its content inside (Fig. S18). To recognize code in microcapsule automatically, we programmed code recognition system with Matlab. First, in microwell image, each microcapsule is recognized by fluorescence difference (Fig. S19A). Each image of microcapsule is then processed by barcode or character code recognition program. In barcode, by measuring thickness of each bar line, we can obtain correct code in microcapsule (Fig. S19B). In character code, align mark is added under the character to help rotation of image. By utilizing conventional optical character recognition (OCR) program, we can successfully recognize character code (Fig. S19C).

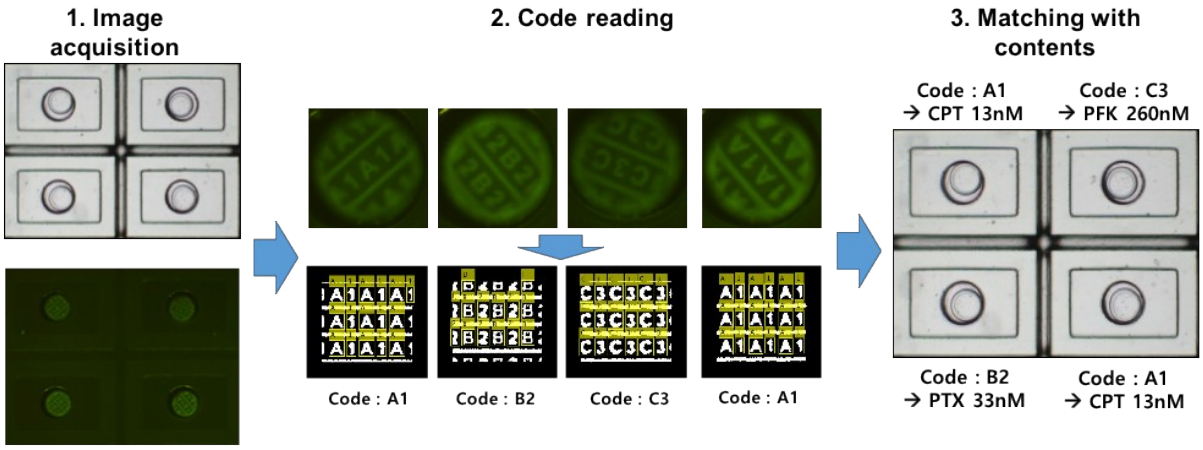

Fig. S18. Process for matching microcapsules with their contents

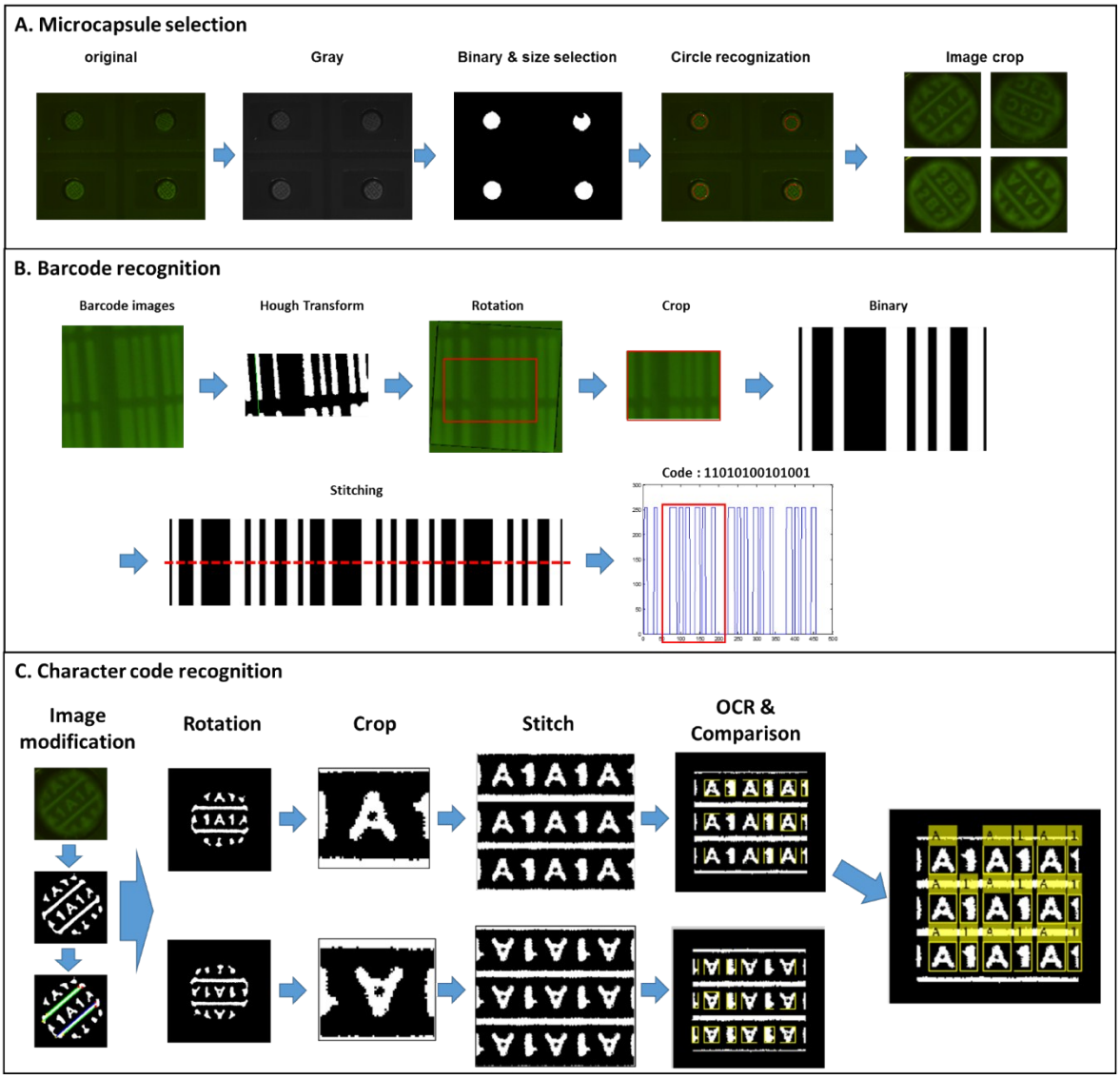

Fig. S19. Image processing for code recognition. (A) In microwell image, each microcapsule is recognized by fluorescence difference. Each identified image of microcapsule is recognized

by (B) barcode recognition process or (C) character code recognition process.

Image processing for apoptotic cell counting

To analyze drug-induced apoptosis results in conventional 96-well plate and our microwell array, we adopted CellC algorithm to count apoptosis cells through fluorescent images[3]. In the results of 96-well plate, fluorescent images of apoptotic cells in each well are directly analyzed with the algorithm. In the results of microwell array, we first obtained fluorescent images of microwell array in 4X microscope objective view (Fig. S20 A). Each 4X image is cropped to obtain region of interest (Fig. S20 A, B, red rectangle) and modified to remove any fluorescence noise coming from microcapsules, which can interrupt counting of apoptotic and total cells. Here, we replaced the area where the microcapsules are assembled with background color of microwell. (Fig. S20 A, B, orange circle). After that, all microwells were analyzed by cell counting software to count the number of apoptotic cells and total cells (Fig. S20 C).

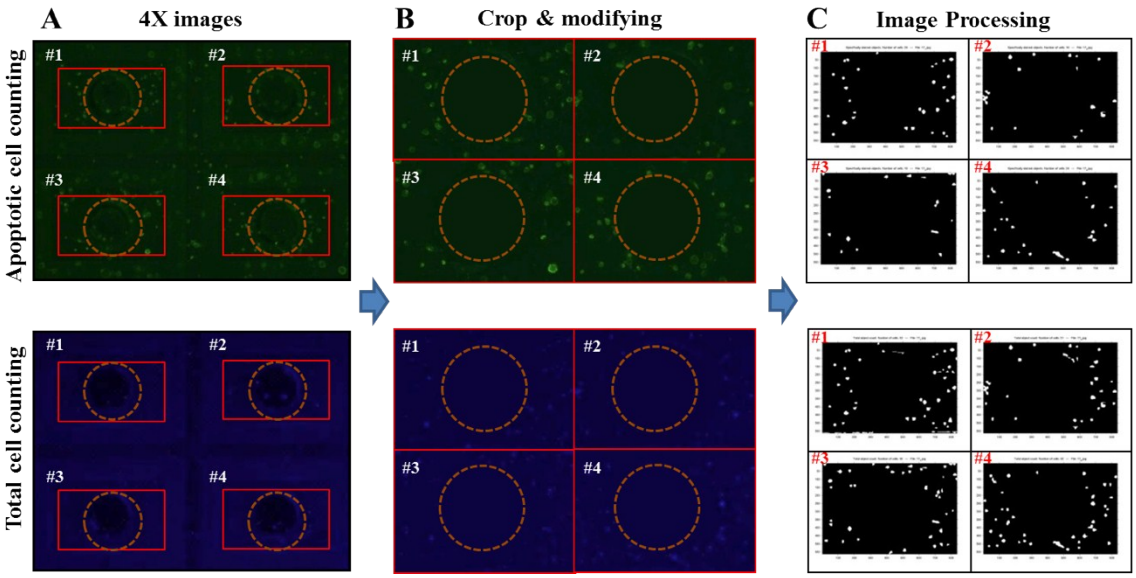

Fig. S20. Image processing for apoptotic cell counting. (A) The 4X magnitude images are cropped to obtain regions of interest. (B) Each cropped image is further processed to remove background fluorescence noise. (C) The images were analyzed by cell counting software to count the number of the apoptotic cells.

Supplementary movies

Movie S1: Assembly of the microcapsules in microwell array.

This is a movie showing assembly of heterogeneous microcapsules in microwell array. Heterogeneous microcapsules are dispensed into microwell array and swept by planar substrate

such as slide glass or cell scrapper. By a few sweeping processes, microcapsules are assembled in almost every microwell. After assembly, extra microcapsules are easily removed from microwell by additional sweeping processes.

Movie S2: Double emulsion droplet generation.

This is a movie showing generation of double emulsion droplet. The water/PFPE/water double emulsion droplets are generated in PDMS microfluidic channel with selective hydrophilic coating. After generation of double emulsion droplet, the double emulsion droplets are exposed to UV light. The PFPE monomer is then polymerized and the core liquid is encapsulated by a solid PFPE shell.

Movie S3: High physical flexibility of the microcapsule.

This is a movie showing the high physical flexibility of microcapsule. After polymerization of liquid microcapsules, the microcapsules are pressed by tweezers to identify its stability. Because of high elastic modulus of PFPE polymer, the microcapsules can withstand external force to a certain extent and the liquid inside microcapsule can be encapsulated stably without leakage and cross-contamination.

Movie S4: Three-dimensional feature of the encoded microcapsule.

This is a movie showing three-dimensional feature of encoded microcapsules measured by confocal microscope. From this movie, we can verify that the code patterns only exist on the PFPE shell region.

Movie S5: Automatic releasing process using motorized stage.

This is a movie showing automated breakage process using motorized stage. After pushing and fixing ball bearing on the micropillar array, the motorized stage moves to scan whole area of microwells. As micropillar which is forced by ball bearing squeeze each microcapsule, the microcapsule is ruptured and core liquid is released.

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