Supporting Information

An automated droplet-based microfluidic platform for multiplexed analysis of biochemical markers in small volumes

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❖ Supplementary Materials and Methods

S1. Materials

4-Aminoantipyrine (4-AAP), 3α-hydroxysteroid dehydrogenase (HSD), β-D-glucose, glucose oxidase from Aspergillus Niger (GOx), L-lactate dehydrogenase (LDH), β-nicotinamide adenine dinucleotide sodium salt (NAD^+), sodium L-lactate, palmitic acid, resazurin sodium salt, sodium glycochenodeoxycholate, Tetramethylrhodamine isothiocyanate (TRITC)–dextran and tunicamycin were purchased from Sigma-Aldrich Chemicals (Milwaukee, WI, USA). Diaphorase (DPR) and horseradish peroxidase (HRP) were obtained from MP Biomedical (Santa Ana, CA, USA). N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methoxyaniline sodium salt (ADOS) was purchased from Dojindo Molecular Technology (Washington, D.C., USA). Sylgard®184 silicone elastomer kit (PDMS) for polydimethylsiloxane curing was purchased from Dow Corning (Midland, MI, USA). SU-8 2025 negative photoresist, SU-8 2100 negative photoresist, AZ9260 positive photoresist, SU-8 developer and AZ-400K developer were obtained from MicroChem (Westborough, MA, USA).

S2. Fabrication of droplet-generating microfluidic devices

To fabricate the flow layer mold, AZ 9260 photoresist was spin-coated onto a 4 in. silicon wafer at 800 rpm for 35 s, yielding a height of 18 μm, soft-baked at 110°C for 3 min, exposed to UV light through a photomask, and developed in AZ-400K developer. Next, the patterned silicon wafer was thermally reflowed at 135°C for 30 min to transform the rectangular channels into a round-shaped flow channel, followed by a hard-bake at 190°C for 120 min. Subsequently, SU-8 2025 was spin-coated at 1000 rpm for 30 s to form 60-μm thick layer of photoresist. This layer included only the oil-phase flow channels. Then, the silicon wafer was soft-baked at 65°C for 3 min and at 95°C for 9 min, exposed to UV light with its respective photomask, developed on SU-8 developer, and hard-baked at 150°C for 30 min For the control mold, SU-8 2010 photoresist was spin-coated on a silicon wafer at 1000 rpm for 30 s to achieve a thickness of 20 μm. It was then soft-baked at 65°C for 5 min, patterned by UV expo-sure with a photomask, developed in SU-8 developer, and hard-baked at 150°C for 30 min. Finally, the two molds were exposed to chlorotrimethylsilane for 30 min in a vacuum desiccator.

To replicate SU-8 mold of the droplet-generating microfluidic device, a PDMS mixture (20:1 weight ratio, curing agent to prepolymer) was spin-coated on top of the flow layer-mold at 1000 rpm for 30 s to create a ~100-μm thick layer, while a mixture of PDMS (5:1 ratio) was poured on the control-layer mold. Both molds were degassed in vacuum for 15 min, followed by a baking step at 80°C for 25 min. Next, the control layer was peeled off, devices were cut out, and access holes punched out. The control-layer replicas were manually aligned over the flow layer mold where the flow layer was still attached to the mold. Then, both layers were baked together for 90 min at 80°C. The resulting multilayer devices were peeled off from the flow mold and holes were punched out. Finally, the devices were bonded to a microscope slide using oxygen plasma treatment.

S3. Fabrication of microfluidic devices for cultivation of hepatocyte spheroids

The molds of the devices for cultivation of hepatocytes were fabricated using SU-8 2100. To construct the flow layer (100 μm in thickness), an SU-8 2100 layer was spin-coated on the silicon wafer at 3500 rpm for 30 sec. After soft-baking at 65 °C for 5 min and at 95 °C for 20 min, the wafer was

exposed to UV through a photomask and then developed in SU-8 developer. To fabricate the layer containing microwells we followed a similar protocol except that thickness of the SU-8 layer was 500 μm. Finally, all the molds were hard-baked at 150 °C for 30 min.

PDMS slabs containing flow channel and microwells were fabricated by pouring a 10:1 mixture of PDMS elastomer and curing agent onto a SU-8 mold, degassing in a vacuum chamber for 30 min and baking at 80°C for 90 min. The resulting PDMS replicas were peeled off from the molds, cut out and punched to create inlet/outlet ports. Finally, the layers were manually aligned and bonded by treatment with oxygen plasma.[1,2] The resulting microfluidic devices were sterilized via UV irradiation for 30 min and then washed with sterile DPBS. The chamber of the cell culture device was incubated with sterile 0.2% (w/v) Pluronic® F127 at 4°C for 12 h to generate a non-fouling surface. Finally, the micro-fluidic devices were washed with sterile DPBS three times and stored at 4°C until use.

S4. Cultivation of hepatocytes in microfluidic devices

Primary rat hepatocytes were isolated from adult female Lewis rats weighing 110-200 g (Charles River Laboratories, Boston, MA, USA) using a two-step collagenase perfusion procedure.[3] After isolation, hepatocytes were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplement with fetal bovine serum (10% in volume ratio, Invitrogen), penicillin (200 U/mL, Invitrogen), streptomycin (200 mg/mL, Invitrogen), hydrocortisone sodium succinate (7.5 mg/mL, Sigma), epidermal growth factor (20 ng/mL, Invitrogen), glucagon (7 ng/mL, Sigma), and human insulin (0.5 U/mL, Novolin N). Henceforth, we refer to this culture media as CH media (complete with hormones). Typical viability of isolated hepatocytes was >90% based on a trypan blue exclusion assay.

To seed hepatocytes, cell suspension (250 μL at 3×10^6 cells/mL) was introduced into a cloning cylinder serving as an inlet, with cells pushed into the culture chamber by hydrostatic pressure. After filling all the microwells, the devices were washed with fresh media to remove all the cells outside of the microwells. At 12 hours of culture, the hepatocytes began to aggregate and form spheroids. After 24 h, excess cells were removed from cloning cylinders by pipetting.

S5. Operation of the automated microfluidic bioanalysis module

The developed droplet bioanalysis module was controlled via a custom-made pneumatic system that comprises ten 3-way solenoid valves (model MH1, Festo, Germany), an Arduino Mega 2560 (Arduino, USA), flow regulators and manometers. The pneumatic system is controlled from a PC with a LabVIEW interface (National Instruments, USA). During experiments, the temperature and CO_2 were maintained at 37 °C and 5%, respectively. The control layer microchannels were filled with PBS and the valves were pressurized at 25 psi. Prior to injection of mineral oil and aqueous solutions, microfluidic devices were treated with Aquapel solution (Pittsburgh Glass Works, LLC, USA) for 5 minutes to make channels hydrophobic. Images of fluorescent droplets were acquired using excitation/emission filter set at 530-550/575-625 nm. Visible color in the droplets was monitored using color CCD camera set to 100 μs exposure time.

S6. Preparation of reagent solutions for biochemical assays

The assay solution for colorimetric detection of glucose was prepared by dis-solving GOx (70 U/mL), HRP (117 U/mL), ADOS (3.6 mM) and 4-AAP (3.1 mM) in 1X Dulbecco's phosphate buffered

saline (DPBS, Gibco, pH 7.4). The fluorescence-based LDH assay was prepared by dissolving L-lactate (142.8 mM), NAD^+ (7 mM), DPR (8 U/mL) and resazurin (56.7 μM) in 1X DPBS. The fluorescence assay for measuring bile acids was prepared by dissolving HSD (25 U/mL), NAD^+ (11.7 mM), DPR (40 U/mL) and resazurin (30 μM) in 1X DPBS. The positive control sample for cell-injury monitoring was prepared by mixing LDH (75 U/L) and sodium glycochenodeoxycholate (150 μM) in CH media containing 25 mM glucose. The assay reagent solutions and positive control samples were freshly prepared prior to each experiment.

S7. Assessment of cross-contaminations among biochemical assays

To assess the cross-contaminations among employed assays, three reference solutions containing known high concentration of glucose (25 mM) or LDH (75 U/L) or bile acid (150 μM). These solutions were dispended in triplicates into wells of a 384-well microtiter plate, 20 μL per well. Subsequently, we added equal volume of assay reagents for detection of glucose, LDH and bile acids. The composition of each assay reagent for glucose, LDH and bile acid were same with that of assay reagents used in droplet platform (See Supplementary Materials and Methods S6). After 5 min of incubation, the absorption (for glucose assay) and emission (for LDH and bile acid assays) spectra were monitored using spectrophotometer. Also, the microscopic images of each well were registered using fluorescence microscope. Color bright field imaging was used for glucose assay and fluorescent imaging (TRITC filter) was applied to LDH and bile acid assay.

S8. Calibration curve in the microfluidic device

To construct calibration curves in the device, four different concentrations of analyte (glucose, bile acid or LDH) and one mix of assay components were injected into five separate channels. For each analyte concentration, five droplets were generated for statistical analysis. After generating droplets containing a mixture of a standard analyte concentration and assay components, the flow was stopped for 15 min to allow time for enzymatic reaction to take place. For glucose detection, absorbance measurements were made using a microscope-mounted color camera set to 100 μs of exposure time. For LDH and bile acid assays, red fluorescence filter was used with a 250 ms of exposure time. The images of droplets were taken every 1 min for 15 min to monitor time course of reaction.

❖ Supplementary Figures

Figure S1

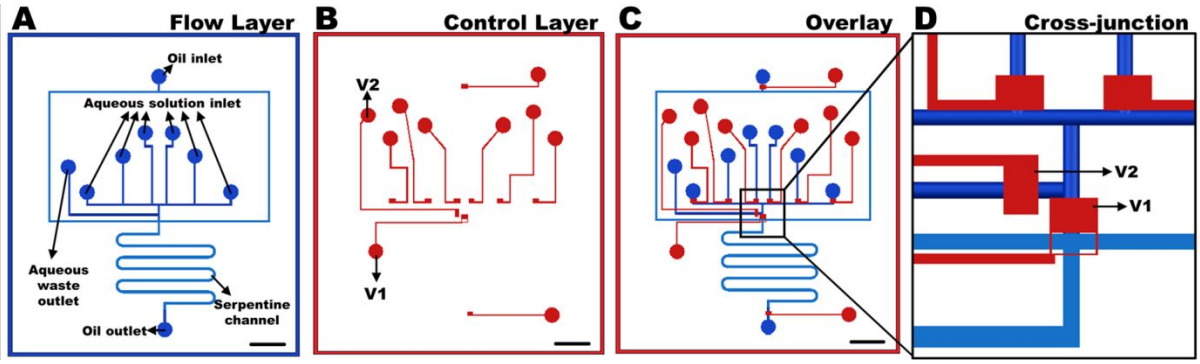

Figure S1. CAD design of photomasks for droplet device fabrication. (A) CAD design for flow layer photomask. (B) CAD design for control layer photomask. (C) Merged image of flow layer mask and control layer mask. (D) Enlarged image of cross-junction (droplet generating site) on (C). Scale bar: 1 mm.

Figure S2

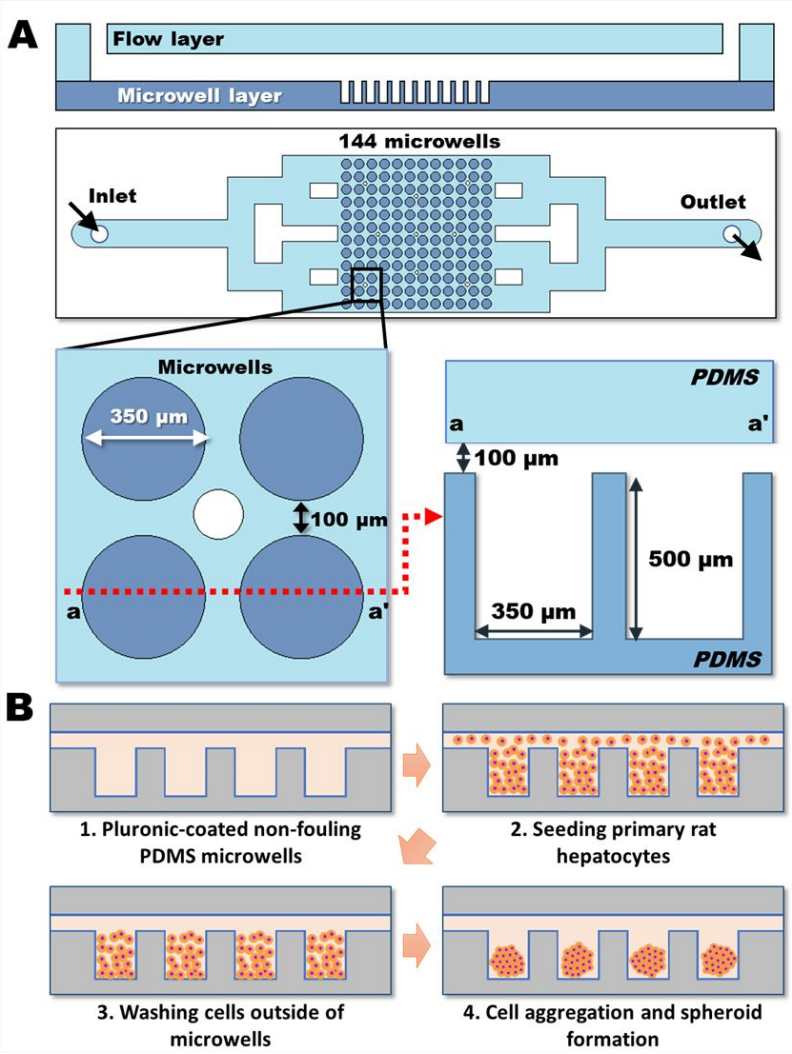

Figure S2. Schematic illustrations for design of hepatocyte culturing microfluidic device and procedure of hepatocyte spheroid forming. (A) Structure of hepatocyte culturing device. The hepatocyte culturing device is composed of micro-well array layer and flow channel. The depth of microfluidic channel in flow layer is 100 μm. The microwell layer contains 144 circular microwells. The width and depth of each microwell were 350 μm and 500 μm, respectively. (B) Cartoon of procedure for hepatocyte spheroid forming. Prior to cell seeding, PDMS surface of culturing device was treated with Pluronic to construct non-fouling surface. To the non-fouling treated microwells, primary rat hepatocyte suspension (250 μL at 3×10^6 cells/mL) was introduced. After the cell settling down, excessive cells outside of wells were removed. Then, the hepatocyte-seeded device was incubated at 37 °C with 5% CO_2 condition. After 12h from the seeding, the primary rat hepatocytes in each microwells form spheroids.

Figure S3

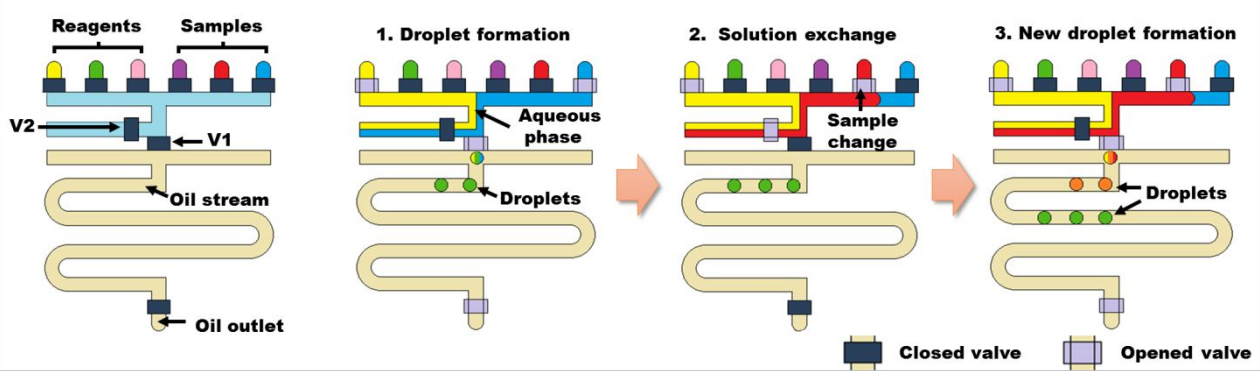

Figure S3. Cartoons represent the droplet generation procedures. (Far-left panel) The flow layer has six aqueous solution injection ports and it is connected to long oil-phase microfluidic channel. Three different biochemical assay reagents were connected into three aqueous inlet ports on left-handed side and three different sample solutions were connected to three inlets on right-handed side. By the closed pneumatic valves on each inlet port, each solution (reagents and samples) cannot be injected to flow channel. When the valves on yellow reagent and blue sample are opened, those aqueous solutions will be introduced into flow channel. Under the V1-closed and V2-opened condition, the laminar aqueous streams bypassed to aqueous outlet. When on-off states of V1 and V2 are rapidly switched, the aqueous stream intersects into oil phase stream and forms droplet (green color). After the generation of first droplet batch, the blue sample can be introduced into flow channel instead of red sample by operating pneumatic valves on sample inlets. Then, new droplets (scarlet color) can be generated with new sample by operating V1 and V2 in same way for green droplet generation.

Figure S4

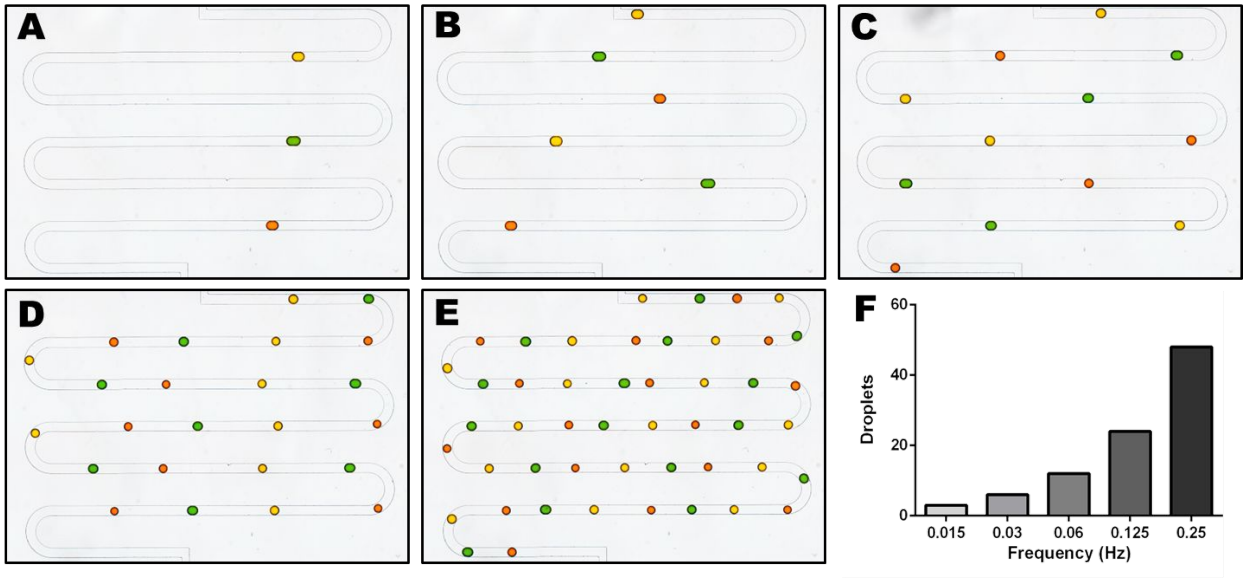

Figure S4. Control of droplet numbers by varying V1 operation frequency. (A) Droplet generation result under V1 operation frequency condition at 0.015 Hz. (B) Droplet generation result under V1 operation frequency condition at 0.03 Hz. (C) Droplet generation result under V1 operation frequency condition at 0.06 Hz. (D) Droplet generation result under V1 operation frequency condition at 0.125 Hz. (E) Droplet generation result under V1 operation frequency condition at 0.25 Hz. (F) Correlation between droplet numbers and V1 valve operation frequency. According to the increase of valve operation frequency, number of droplet increases.

Figure S5

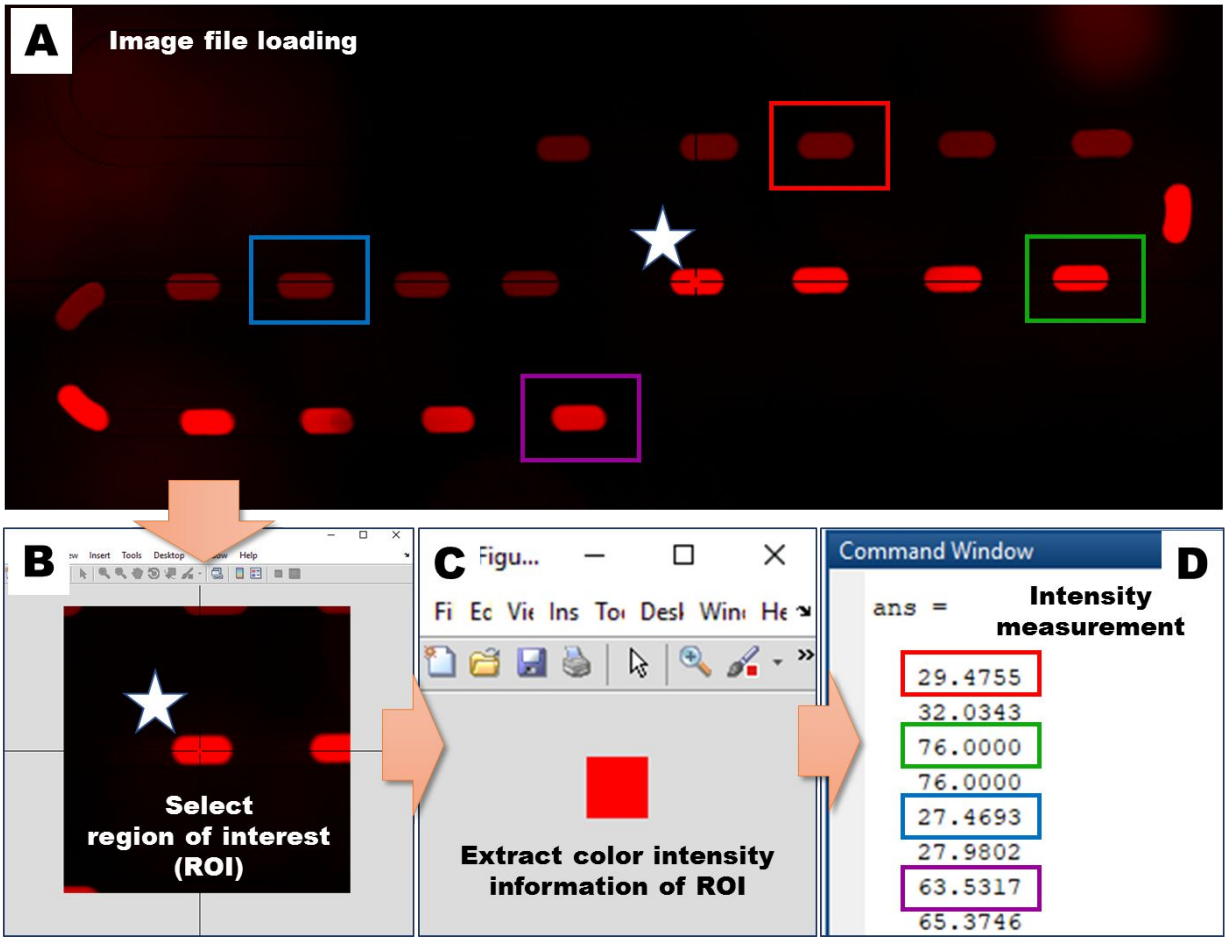

Figure S5. Workflow of optical signal measurement using MATLAB-based custom-made image analysis tool. (A) Image file loading on image analysis tool. (B) Select region of interest (ROI). (C) Extraction of color information on ROI. (D) Measurement of optical signal (e.g., color intensity or fluorescence intensity).

Figure S6

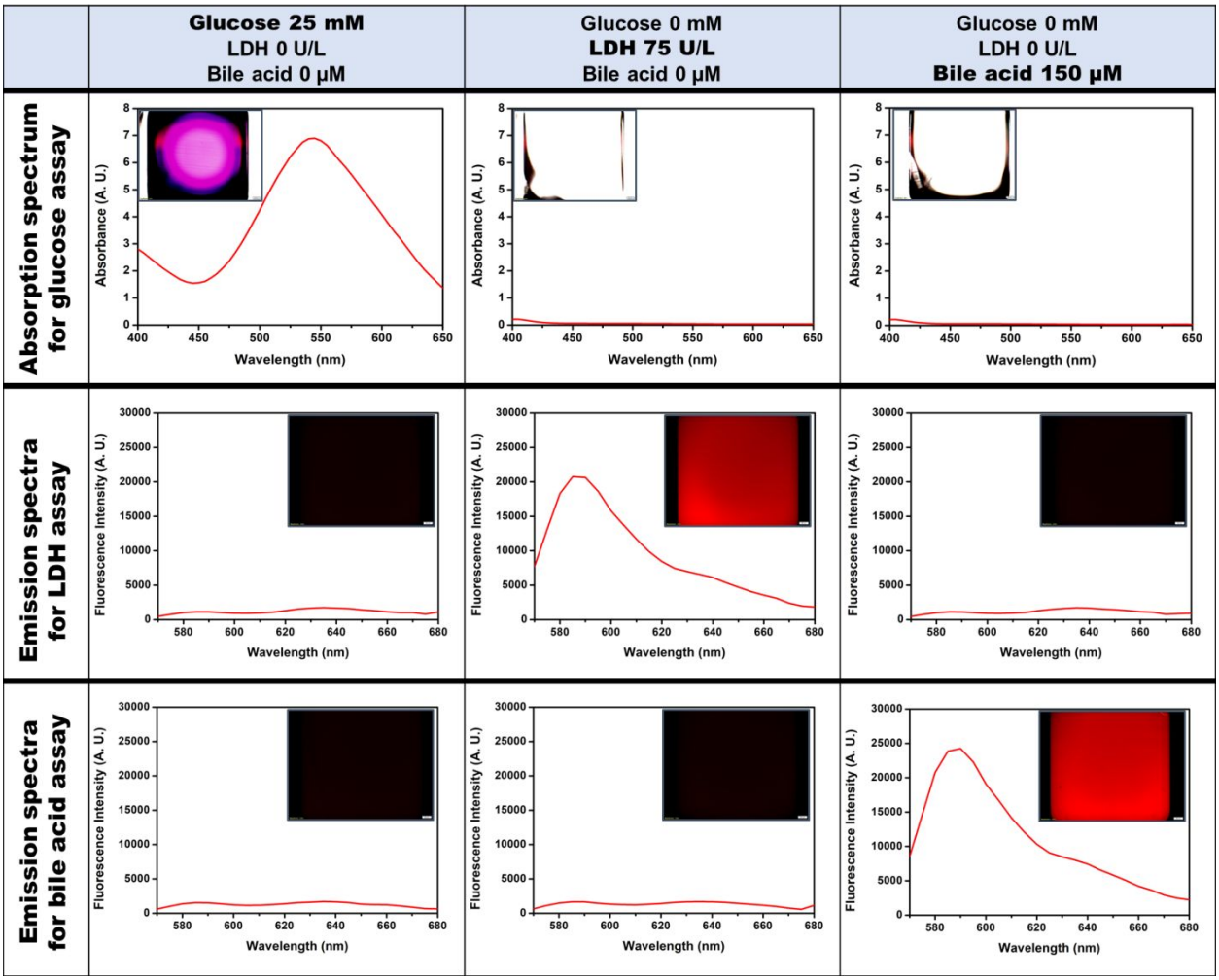

Figure S6. Cross-reactivity demonstration results for three biochemical assays. Top three panels show glucose assay results in spectral analysis and bright field microscopic analysis for glucose sample, LDH sample and bile acid assay sample. Color development and absorbance rising were observed only on glucose sample. Three panels in middle show LDH assay results in spectral analysis and fluorescent (TRITC filter) microscopic analysis for glucose sample, LDH sample and bile acid assay sample. Red fluorescent emission was observed only on LDH sample. Bottom three panels show bile acid assay results in spectral analysis and fluorescent (TRITC filter) microscopic analysis. Red fluorescent emission was observed only on bile acid sample. Inset photographs show the bright-field microscopic images (for glucose assay) and fluorescent microscopic images (for LDH and bile acid assays) of each well.

Figure S7

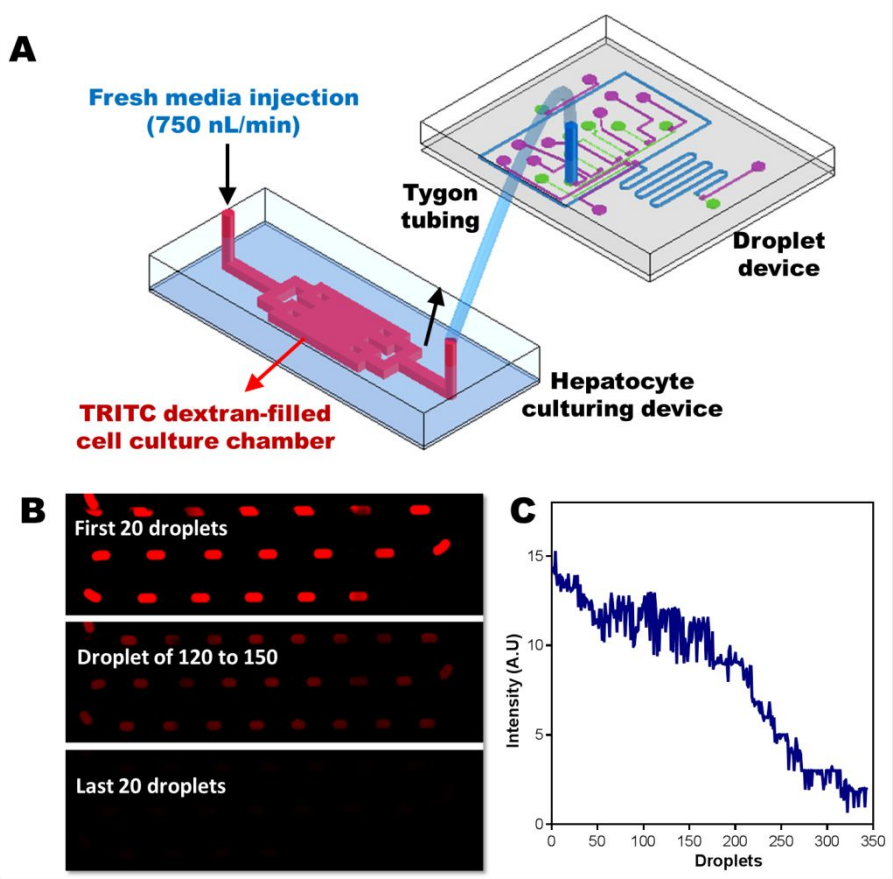

Figure S7. Assessment of dilution in cell culture media sample by the injection of fresh media. (A) Schematic illustration for experimental setup. The hepatocyte culturing device was filled with TRITC dextran solution (70 kDa, 0.5 mg/mL). Then, the cell culture device was connected to droplet device by empty Tygon tubing. By injecting fresh CH media through inlet port of cell culture device, TRITC-dextran solution in cell culturing chamber was transferred to droplet device and formed droplet. During the generation of 350 droplets, their fluorescence intensity changes were monitored by fluorescence microscope. (B) Result images of first 20 droplets (droplet number 1-20, upper panel), middle 20 droplets (droplet number 120-140, middle panel), and last 20 droplets (droplet number 330-350, bottom panel). Compared to droplets from front-end of TRITC-dextran stream, the fluorescence intensity of droplets from rear-end of TRITC-dextran solution was weaker. The decreased fluorescence intensity can be construed as the TRITC-dextran solution at rear-end was diluted by following CH media. (C) Correlation graph between droplet number and fluorescence intensity. The average signal decrease rate of first 30 droplets was less than 10%.

❖ Supplementary Video

Video S1. Droplet generation using various food dye solutions. The automated pneumatic valve controller allows facile change of solution source to be injected and generation of droplets in various colors. These droplets in various colors can be considered as reactants of multiplexed biochemical assays.

❖ References

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(2) Chen, Y.; Zhang, B.; Feng, H.; Shu, W.; Chen, G. Y.; Zhong, J. F. *Lab Chip* **2012**, *12*, 3930-3935.

(3) Dunn J. C.; Yarmush M. L.; Koebe H. G.; Tompkins R. G. *FASEB Journal* **1989**, *3*, 174-177.