A Next-generation Approach

Droplet-based Technologies

Cutting-edge Studies on

Single Cells × Screening

Created by the World of Water Droplets

On-chip Biotechnologies Co., Ltd.

Introduction

Since each cell is unique genetically and phenotypically, it is becoming increasingly important to screen target cells from a bunch of population and to perform Single-Cell Analysis to characterize their individual functions. Their applications range widely from single-cell RNA-seq, which is the most popular, to screening strains that produce high levels of enzymes or antibodies, and isolating microorganisms from environmental or intestinal sources.

The use of droplets has been attracting attention in recent years, as high-throughput techniques for culturing, reacting, and sorting thousands to hundreds-of-thousands of cells have become essential. Droplet is a tiny water drop measuring 20–200 μm in diameter that can be broadly classified into two types: water-in-oil (W/O) droplets and gel microdrops (GMDs).

Water-in-oil (W/O) droplets

- Structure: Emulsion in which water droplets (e.g., the medium) are dispersed in oil and stabilized by surfactants.
- Features: Microorganisms/cells grow and metabolites accumulate in each droplet as they are compartmentalized by oil. Addition of substrates allows for detection of metabolites along with the cultivation.
- Use: Microbial culture, screening of environmental microbes/mutant strains, directed evolution of enzymes, etc.

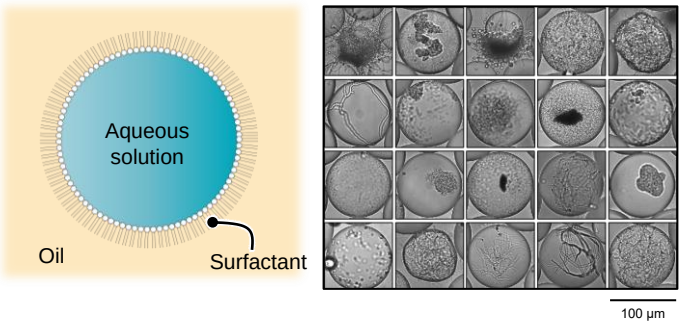

Figure 0-1. Schematic of a W/O droplet (left) and microscopic image of environmental microorganism culture (right).

Related pages ▸▸▸ 01 02 03 04 05

Gel microdrops (GMDs)

- Structure: Droplets with gelled interior.
- Features: Substances are highly permeable both inside and outside the droplet structure because the droplets can be dispersed in an outer aqueous phase, such as a culture medium, rather than in oil, while relatively large materials such as cells, beads, and genomes are retained by the gel as a scaffold.
- Use: Cell culture, screening of mutant strains /antibody-producing cells, microbial interaction studies, etc.

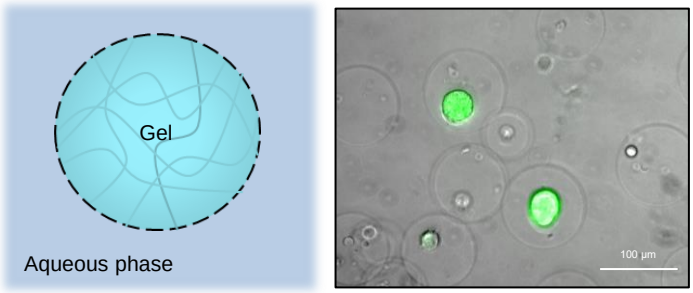

Figure 0-2. Schematic of a GMD (left) and microscopic image of cancer cell culture (right)

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▸ Benefits of droplets

1. High-Throughput: Single-cell encapsulated droplets are generated with an efficiency of **a million or more within 10 minutes**.
2. Downsizing: The volume of droplets is very small, thus **saving scarce reagents**. In addition, several million droplets can be **stored in a single microtube**.
3. Small Bioreactor: Each droplet functions as a reactor/incubator, enabling **single-cell-level enzyme reactions, quantification of secreted products**, etc.

Droplets have been introduced in many fields—including high-throughput screening—as a tool that enables single-cell analysis with higher efficiency than ever before. This booklet contains a variety of examples to provide a broad and deep understanding of the overall landscape of droplets.

CONTENTS

01 Establishment of a next-generation culture method aiming for the isolation of microbial dark matter based on droplet technology

Dr. Naohiro Noda
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A wide variety of microorganisms live in the environment, but it is said that more than 99% of them have not yet been successfully cultured. Traditionally, environmental microorganisms have been cultured using the agar plate method and the limiting dilution method, but the former tends to eliminate slow-growing microorganisms because fast-growing ones are preferentially grown, while the latter method is laborious and time-consuming. In recent years, microfluidic droplet technology has attracted attention as a way to solve these problems by enabling the culture of several million species of microorganisms, each one in its own small, separate space.

A major challenge in obtaining pure cultures of environmental microorganisms has been detecting and isolating droplets containing microorganisms with various growth rates from millions of droplets. To solve this problem, Dr. Noda and his team at the National Institute of Advanced Industrial Science and Technology (AIST) developed FNAP-sort (fluorescent nucleic acid probe in droplets for bacterial sorting), a method that utilizes fluorescence to detect droplets containing grown microorganisms. In this method, a fluorescent nucleic acid probe, which is quenched by FRET (fluorescence resonance energy transfer), is cleaved by RNase generated during microbial growth, resulting in the elimination of FRET and an increase in fluorescence emission (Fig. 1-1A).

As an initial proof-of-principle experiment for FNAP-sort, fluorescent nucleic acid probes, *E. coli*, and LB medium were encapsulated in droplets and incubated (Fig. 1-1B). After one day of incubation, droplets in which *E. coli* had grown emitted strong fluorescence, allowing us to clearly distinguish between fluorescent and non-fluorescent droplets (Fig. 1-1C). The fluorescent droplets containing growing *E. coli* were selectively sorted using On-chip Sort (Fig. 1-1D and E).

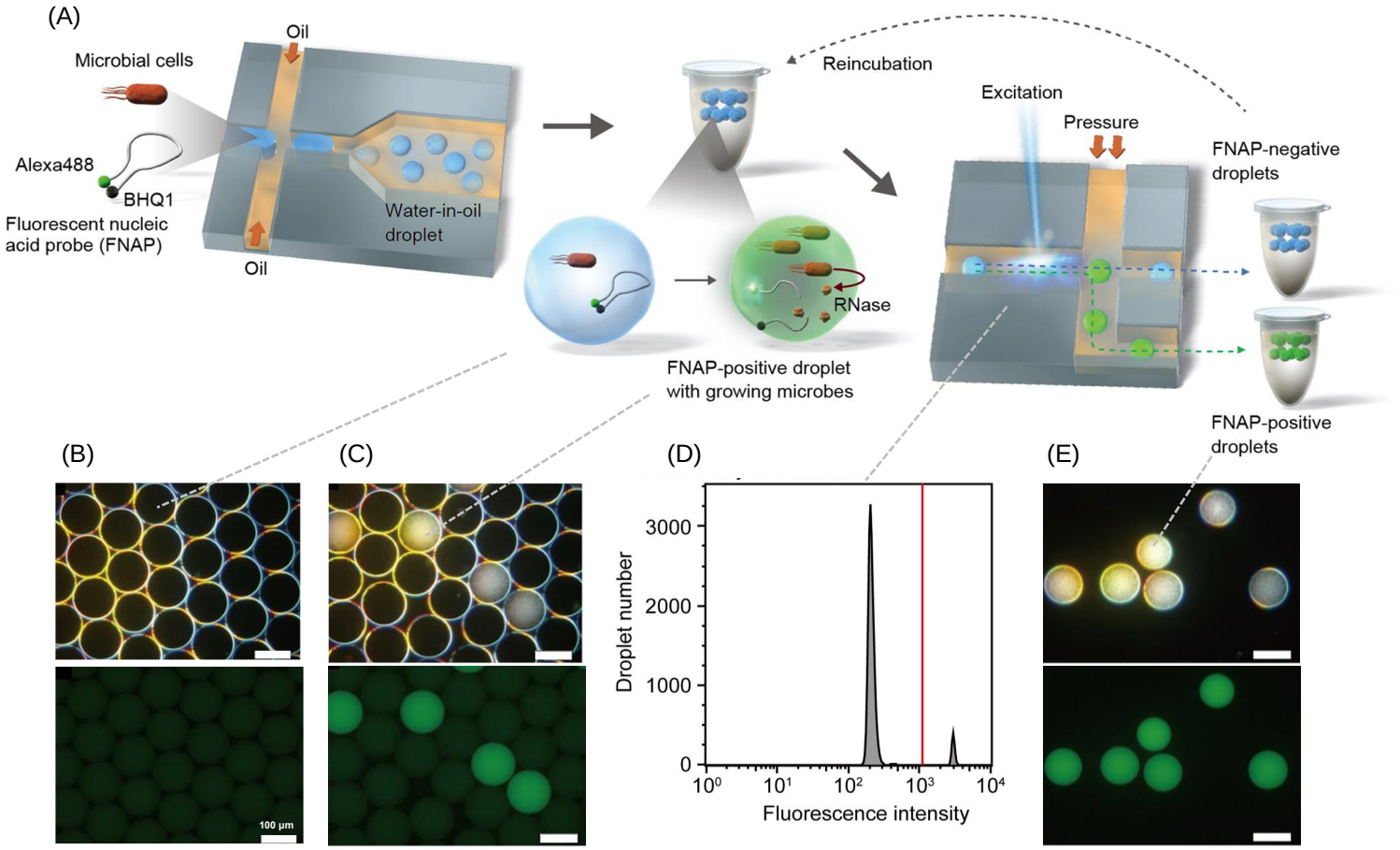

Figure 1-1. FNAP-sort workflow and proof of principle. (A) FNAP-sort workflow, (B) microscopic images of droplets immediately after generation, (C) microscopic images of droplets after one day of incubation, (D) fluorescence histogram of droplets after one day of incubation, and (E) microscopic images of sorted droplets. Upper panels: dark-field observation; lower panels: fluorescence observation for (B), (C), and (E).

Next, a variety of soil microorganisms and fluorescent nucleic acid probes were encapsulated in droplets to perform another FNAP-sort experiment. The microbial species obtained from sorted droplets were different depending on the length of incubation, suggesting that FNAP-sort is useful for sorting droplets containing microorganisms with various growth rates (Fig. 1-2).

These results demonstrated that the combination of On-chip Sort and FNAP-sort enabled efficient verification of microorganism growth and droplet sorting (Fig. 1-3A). In order to isolate and re-culture the sorted microorganisms, sorted fluorescent droplets containing aquatic-derived microorganisms were dispensed one by one into microwell plates using On-chip SPiS (Fig. 1-3B). After dispensing, the microorganisms released from each droplet were scaled up in each well, and growth of the microorganisms was observed in several wells (Fig. 1-3C). Microbiome analysis for each well indicated that microorganisms present at low abundance in the original inoculum were successfully cultured with a purity of more than 99% (Fig. 1-3D: 5-N17, 5-N15, 2-B12, 2-J16, and 2-J3). Furthermore, multiple species of microorganisms were present in some wells, indicating potential for this method as a way to study combinations of symbiotic microorganisms.

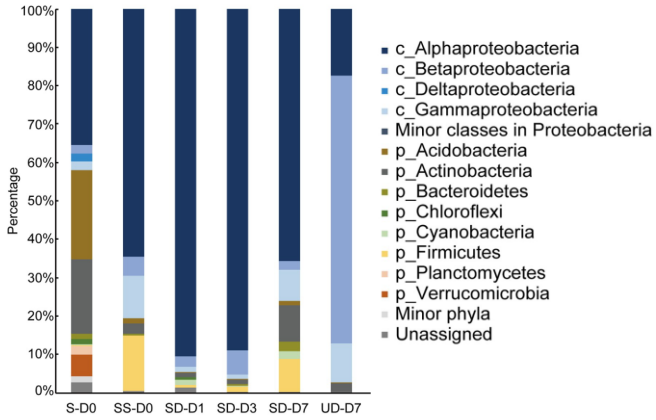

Figure 1-2. Microbiome analysis for soil-derived microorganisms-containing droplets sorted by FNAP-sort.

S-D0: Soil sample, SS-D0: sample just before droplet encapsulation, SD-D1: droplets sorted after one day of incubation, SD-D3: droplets sorted after three days of incubation, SD-D7: droplets sorted after seven days of incubation, UD-D7: droplets not sorted after seven days of incubation.

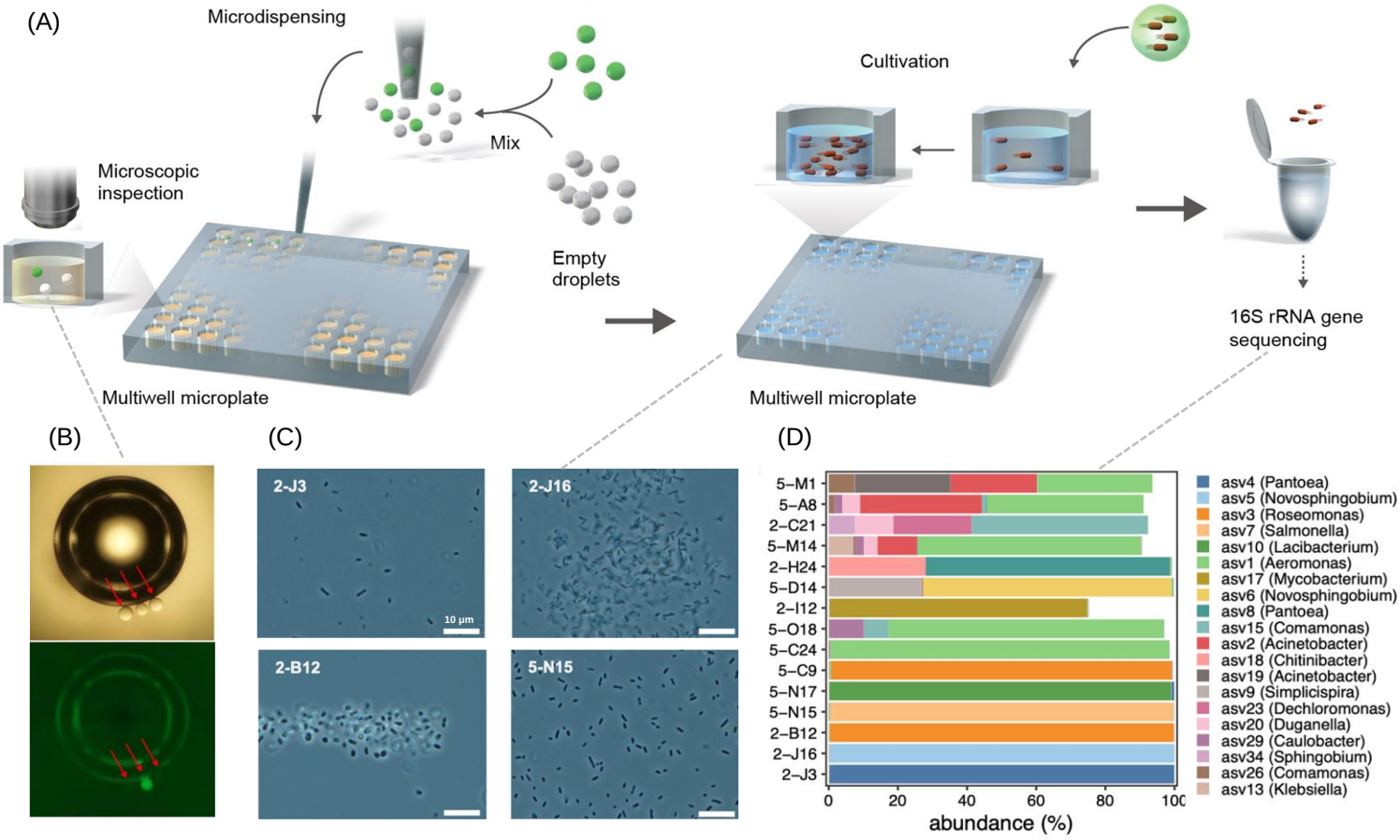

Figure 1-3. Droplet dispensing using On-chip SPiS and scale-up culture of microorganisms derived from individual droplets. (A) Droplet dispensing and analysis workflow, (B) microscopic images of dispensed droplets (upper panel: bright-field observation; lower panel: fluorescence observation), (C) microscopic images after scale-up culture of the dispensed droplets-derived microorganisms, and (D) microbiome analysis of the microorganisms in wells.

In summary, a microbial culture and analysis method that combines FNAP-sort and droplet dispensing have been established and successfully generated highly pure cultures of microbes present at a low abundance in the original source. This method, which can detect microbial growth inside the droplets regardless of the microbial species, is expected to be widely used to culture the uncultured microorganisms.

Reference

Fluorescent nucleic acid probe in droplets for bacterial sorting (FNAP-sort) as a high-throughput screening method for environmental bacteria with various growth rates
Ota, Y., Saito, K., Takagi, T., Matsukura, S., Morita, M., Tsuneda, S., Noda, N.
PLOS One **2019** *14*(4), e0214533.
DOI: 10.1371/journal.pone.0214533 https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0214533

Microdroplet-based system for culturing of environmental microorganisms using FNAP-sort
Saito, K., Ota, Y., Tourlousse, D. M., Matsukura, S., Fujitani, H., Morita, M., Tsuneda, S., Noda, N.
Scientific Report **2021** *11*(1), 9506.
DOI: 10.1038/s41598-021-88974-2 https://www.nature.com/articles/s41598-021-88974-2

02 Evaluation and high-throughput screening of microbial enzymatic activity inside droplets using ACA, a novel hydrophilic fluorescent probe

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Water-in-oil droplets (WODLs) can function as micro-reactors and be used to analyze cellular metabolic activities and enzymatic reactions over time that cannot otherwise be detected by conventional staining of cell surface markers. However, most of the fluorescent probes used to detect these bioactivities are hydrophobic and diffuse from inside the aqueous phase of the WODLs to the external oil phase, limiting their applications for large-scale screening.

To solve this problem, Prof. Ogasawara and his colleagues at Nagaoka University of Technology synthesized 7-aminocoumarin-4-acetic acid (ACA), which is a hydrophilic analog of 7-amino-4-methylcoumarin (AMC), a common fluorescent probe, and then conjugate it with dipeptide to develop a new fluorescent peptide substrate (dipeptidyl-ACA) that can remain inside WODLs for a prolonged period of time. By encapsulating a solution containing dipeptidyl-ACA and microorganisms inside WODLs using On-chip Droplet Generator, dipeptidyl peptidase (DPP) produced by the microorganisms during culture became detectable inside the WODLs. Furthermore, the group showed that, in combination with On-chip Sort, which enables direct sorting of WODLs, this method enables high-throughput screening of microorganisms producing target enzymes (Fig. 2-1A to C).

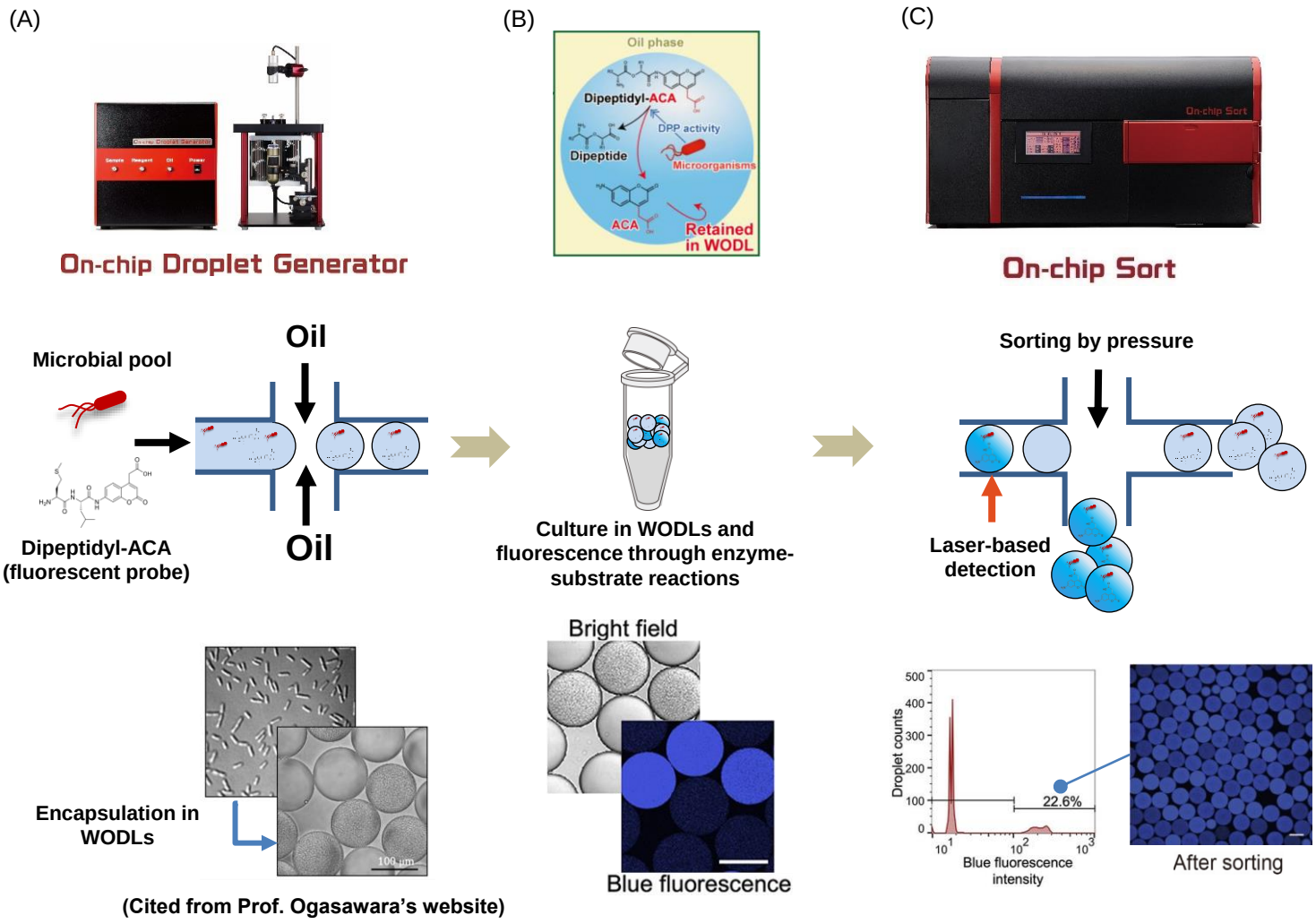

Figure 2-1. Workflow for screening enzyme-producing microorganisms using On-chip Droplet Generator, dipeptidyl-ACA, and On-chip Sort. (A) Generation of WODLs containing dipeptidyl-ACA and microorganisms using On-chip Droplet Generator, (B) an increase in fluorescence intensity due to microbial culture and DPP activity inside WODLs, and (C) detection of fluorescent WODLs using On-chip Sort and sorting-based recovery of microorganisms producing the enzyme at high yields.

Dipeptidyl-ACA enabled the detection of DPP activity produced by *Pseudoxanthomonas mexicana* WO24 in WODLs as blue fluorescence (Fig. 2-2A). Furthermore, sorting with On-chip Sort allows the selective screening of WODLs showing dipeptidase activity from a mixture of red fluorescent microorganisms (Fig. 2-2B) and from microorganisms with high growth potential detected by FNAP (see the previous page) (Fig. 2-2C).

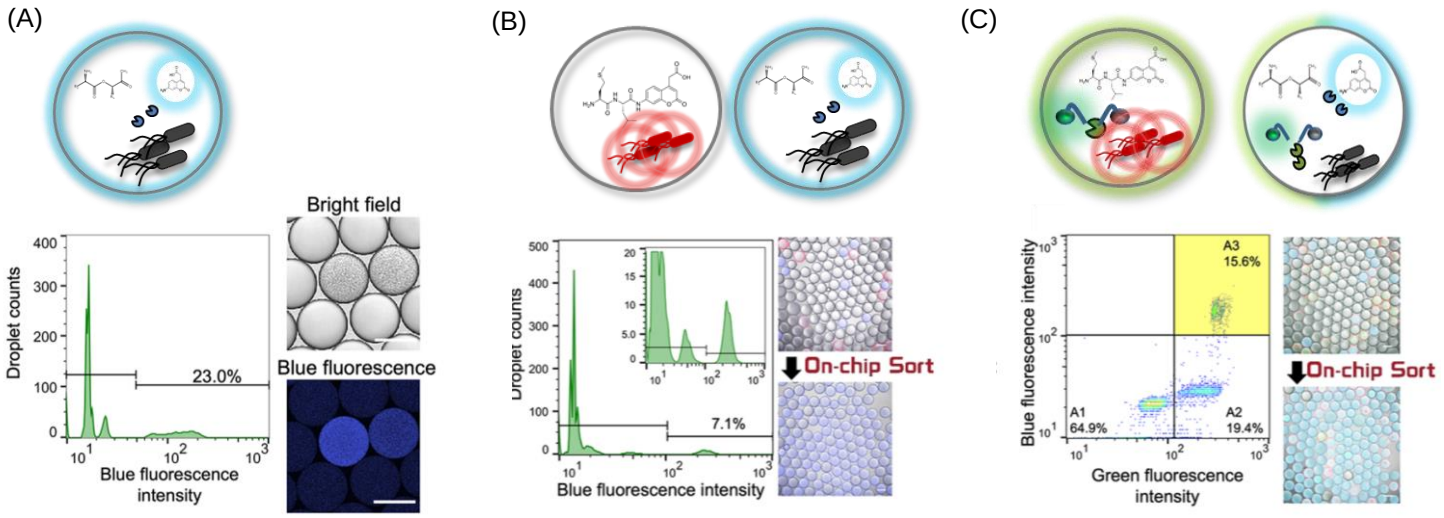

Figure 2-2. Development of a screening system for microorganisms producing the enzyme at high yields using various fluorescent substrates. (A) Detection of DPP from microorganisms using dipeptidyl-ACA, (B) screening of microorganisms producing the enzyme at high yields using dipeptidyl-ACA and On-chip Sort, and (C) detection of microorganisms producing the enzyme at high yields using dipeptidyl-ACA and FNAP and screening with On-chip Sort.

The team conducted a screening of microorganisms in *tofu* factory waste fluid using the following flow diagram (Fig. 2-3A to C) and 16S rRNA gene analysis of the microorganisms in the sample at each screening step, and confirmed that this screening system, using dipeptidyl-ACA as an indicator, enriched the abundance of DPP-producing microorganisms (Fig. 2-3D).

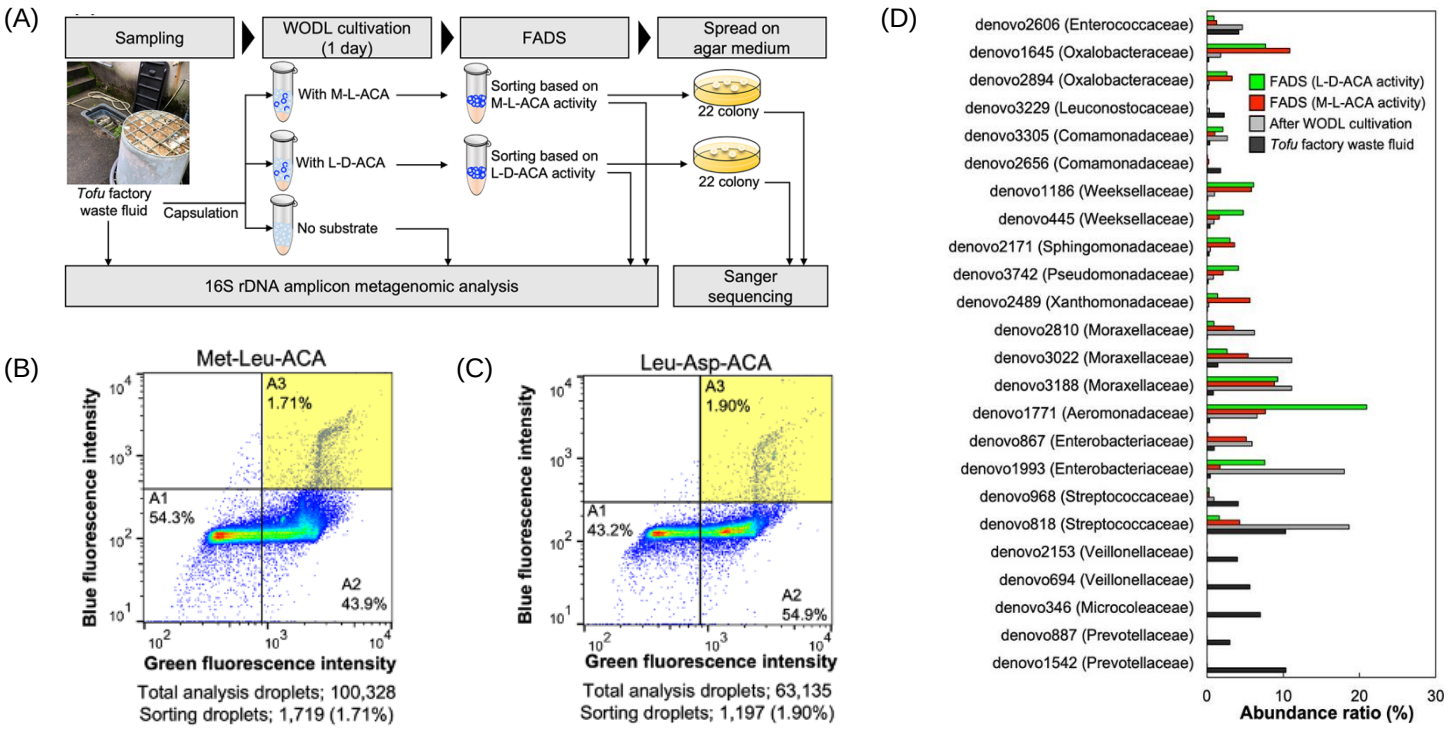

Figure 2-3. An example of high-throughput screening using ACA and FNAP-sort. (A) Workflow diagram of high-throughput screening using DPP activity as an indicator for microorganisms in *tofu* factory waste fluid, (B, C) plots and sorting gates for droplets containing Met-Leu-ACA (B) and Leu-Asp-ACA (C) as substrates and FNAP-sort analyzed with On-chip Sort (the yellow areas (A3) are sorted), and (D) metagenomic analysis based on 16S rRNA genes and the top 10 OTU ratios in the samples before and after sorting (Leu-Asp-ACA: green, Met-Leu-ACA: red).

The combination of ACA, a hydrophilic fluorescent substrate, with WODLs and On-chip Sort enabled high-throughput screening of enzyme-producing microorganisms. This method is expected to make a significant contribution to the discovery of various useful microorganisms.

Reference

7-Aminocoumarin-4-acetic Acid as a Fluorescent Probe for Detecting Bacterial Dipeptidyl Peptidase Activities in Water-in-Oil Droplets and in Bulk
Nakamura, A., Honma, N., Tanaka, Y., Suzuki, Y., Shida, Y., Tsuda, Y., Hidaka, K., Ogasawara, W.
Analytical Chemistry **2022** *94* (5), 2416–2424.
DOI: 10.1021/acs.analchem.1c04108. https://pubs.acs.org/doi/10.1021/acs.analchem.1c04108#

03 Development of a novel transformation method for the filamentous fungus *Trichoderma reesei*

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Filamentous fungi are widely used as hosts for heterologous protein production because they can secrete large amounts of hydrolytic enzymes, bioactive compounds, and even recombinant proteins. The protoplast–PEG method is the most common method for constructing transformants and is utilized by many researchers. However, the selection process using agar media after gene transfer to protoplasts has challenges such as long incubation periods due to cell wall recovery, low regeneration efficiency, and small numbers of candidate strains. Prof. Ogasawara and his team have devised a method to transform a large number of filamentous fungi in a short time period with a higher regeneration efficiency than that of agar media by adopting a selection method that utilizes a droplet-based cultivation.

First, conditions for the droplet-based cultivation of filamentous fungi were determined using *Trichoderma reesei* as a model. DNA fragments that express GFP driven by the cbh1 promoter were used for transformation. The cbh1 promoter is regulated in the presence of sugar, and the team found that expression was best induced in the presence of sophorose in the droplet-based cultivation. Thus, transformed *T. reesei* was cultured in the presence of sophorose, resulting in mycelial growth observed after 12 hours of cultivation, and GFP-derived green fluorescence was observed within the mycelium after 18–24 hours (Figure 3-1). Mycelia were retained inside the droplets after 24 hours of cultivation, but after 36 or 48 hours, mycelia penetrated into the external oil phase, and fusion between droplets was observed.

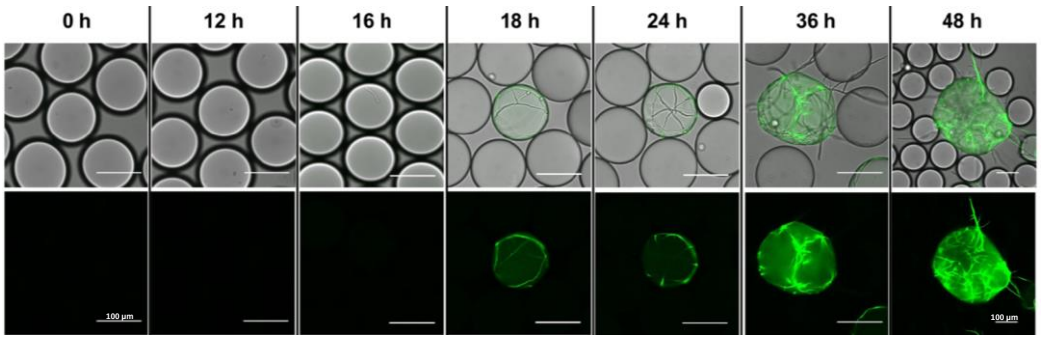

Figure 3-1. Droplet-based culture of GFP-expressing filamentous fungi (top: superimposed, bottom: fluorescent).

Next, the team observed the regeneration rates of protoplasts with the conventional agar medium method compared to the droplet method. In the droplet method, approximately 100 protoplasts were encapsulated in droplets, and after one, two, or three days of fungal recovery, colonies formed on the agar media and the regeneration rate was measured (Figure 3-2A). The results showed a regeneration rate of 31.62% in the three-day culture, which was more than eight times higher than the agar culture method, indicating extremely high efficiency. Reasons for the high regeneration rate include: 1) no need for high temperature treatment at 42–60°C with the addition of top agar (to dry protoplasts and prevent osmotic pressure fluctuations), 2) fluorinated oil in the droplet-based cultivation environment provides a better oxygen supply than agar, and 3) compartmentalization by droplets prevents nutrient competition among protoplasts. Finally, the team conducted a screening demonstration test of transformants using the droplet method. DNA fragments were transferred into protoplasts, which were then incubated in droplets for 24 hours, resulting in GFP fluorescence in some droplets due to the transformation (Figure 3-2B). Then, 247 droplets with strong fluorescence were sorted out from 200,000 candidate strains. The obtained candidate strains were isolated on agar media and checked by colony PCR, which confirmed that gene cassettes were amplified in 96.6% of the obtained candidate strains.

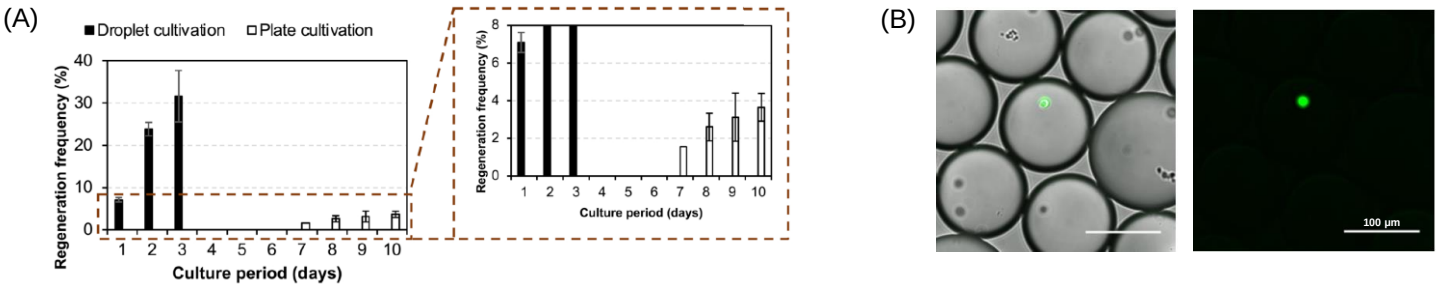

Figure 3-2. Protoplast culture using the droplet method. (A) Protoplast regeneration rates in the droplet and agar medium methods, and (B) microscopic images of transformed protoplasts after 24 hours of cultivation (left: superimposed, right: fluorescent).

Reference

A novel high-throughput approach for transforming filamentous fungi employing a droplet-based microfluidic platform
Luu, X. C., Shida, Y., Suzuki, Y., Sato, N., Nakamura, A., Ogasawara, W.
New Biotechnology **2022** *72*, 149–158.
DOI: 10.1016/j.nbt.2022.11.003 https://doi.org/10.1016/j.nbt.2022.11.003

04 Impact of compartmentalization of cultivation on diversity

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A wide variety of microorganisms inhabit the soil, the hydrosphere, and extreme environments, and it has been estimated that the number of microbial cells on Earth exceeds 10^{30}. In order to study the ecological and physiological functions of microorganisms, it is essential to isolate each species from complex microbial communities. Today, the agar plate method established by Robert Koch in the 1870s is still the most commonly used method to isolate microorganisms. However, in non-compartmentalized environments containing microorganisms with different growth rates such as agar plates and liquid media in flask, fast-growing species would outcompete slow-growing species (Fig. 4-1A: Non-compartmentalized). Since the 2000s, attention has been focused on droplet technology, which divides the culture space into minute compartments, allowing a large number and variety of microorganisms to be cultured while physically separated from one another (Fig. 4-1A: Compartmentalized). What effect does compartmentalization of microorganisms in droplets have on actual culture?

To investigate the effect of spatial compartmentalization of microbial communities, soil microorganisms were cultured in bulk liquid (in the order of milliliters, non-compartmentalized) and droplets (in the order of picoliters, compartmentalized) methods, and the microbiota after seven days cultivation were analyzed using 16S rRNA gene sequencing (Fig. 4-1B). After bulk liquid culture, microorganisms belonging to Proteobacteria were dominant, and the microbiota diversity significantly decreased compared to that of the soil. In contrast, the microbiota after droplet cultivation was found to be more diverse than that of the bulk liquid cultivation and relatively retained the diversity of the soil. These results indicate that compartmentalization allows complex microbial communities to be cultured while maintaining a high level of diversity. This is likely due to slow-growing microorganisms not being eliminated due to the reduction of competition by compartmentalization.

Droplets after culture were grouped together for microbiome analysis, but it is expected that a combination of techniques to isolate droplets one by one will be applied to the acquisition and analysis of single pure cultures in the future.

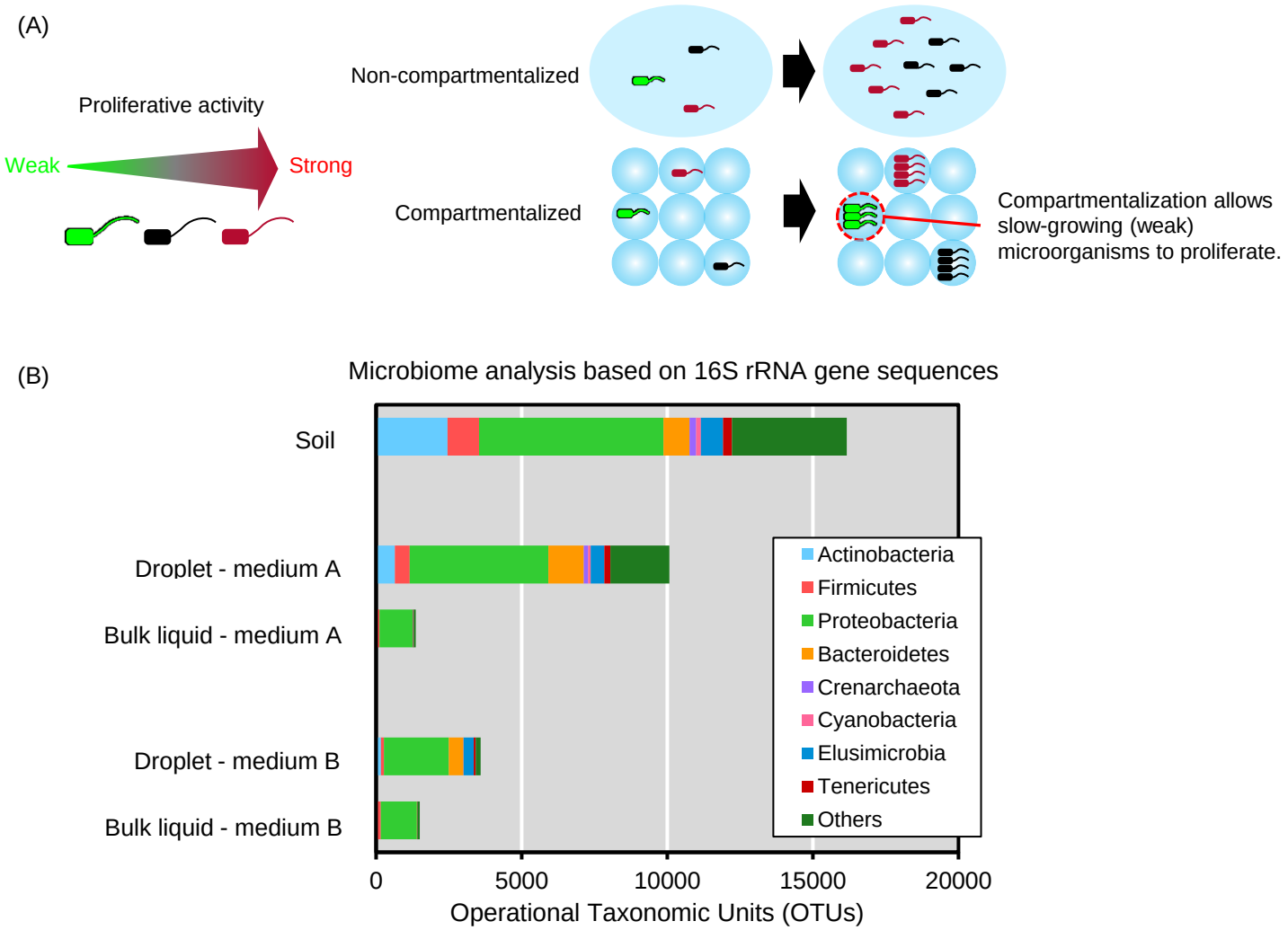

Figure 4-1. Comparison of droplet-based and bulk liquid cultures. (A) Schematic diagram of spatial compartmentalized and non-compartmentalized culture, and (B) microbiome analysis after various types of culture.

05 Large-scale rapid screening method for antagonistic microorganisms against pathogenic bacteria

Dr. Yasunori Ichihashi and Dr. Megumi Narukawa
RIKEN BioResource Research Center

In an effort to address global environmental issues caused by rapid population growth, low environmental-impact agriculture that reduces the use of agrochemicals and chemical fertilizers is attracting attention. Biopesticides that use microorganisms with antagonistic effects against pathogens (antagonistic microorganisms) have been under development in recent years as part of this effort; however, there are some challenges, such as a narrow range of applicable disease-causing species, low field persistence, and a lack of stability in quality. To solve these problems, it is necessary to screen a wider variety of antagonistic microorganisms, but a major bottleneck has been the vast amount of laboratory materials and space, as well as a great deal of time and labor, required.

Drs. Ichihashi and Narukawa at the RIKEN BioResource Center have developed a technology to visualise the antagonistic effects of co-culturing microorganisms and GFP-expressing pathogenic bacteria in droplets as fluorescence decay of the pathogen's fluorescence signal. This technology, which is based on the On-chip Droplet Generator and On-chip Droplet Selector, has succeeded in saving a significant amount of time, labor, experimental materials, space, and cost for screening useful microbes compared to conventional methods (Fig. 5-1).

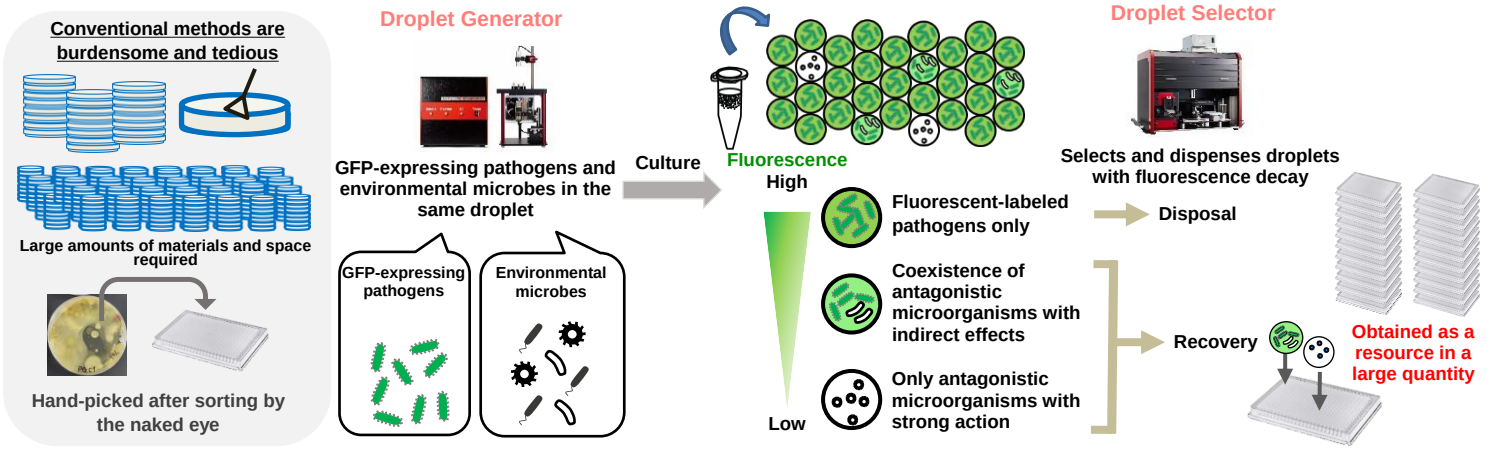

Figure 5-1. Workflow of the screening method for antagonistic microorganisms using droplet technology.

Conventional microbial screening using droplet technology involves dilution of the microbial suspension until the number of microorganisms in the droplet reaches 0–1, resulting in many empty droplets, which reduces throughput. The new technology enables fluorescence detection at a single wavelength by encapsulating multiple pathogens and screening source microorganisms so that no empty droplets are created, thereby dramatically improving throughput. This also enables microorganisms to be screened that cannot be cultured alone but can be cultured by symbiosis.

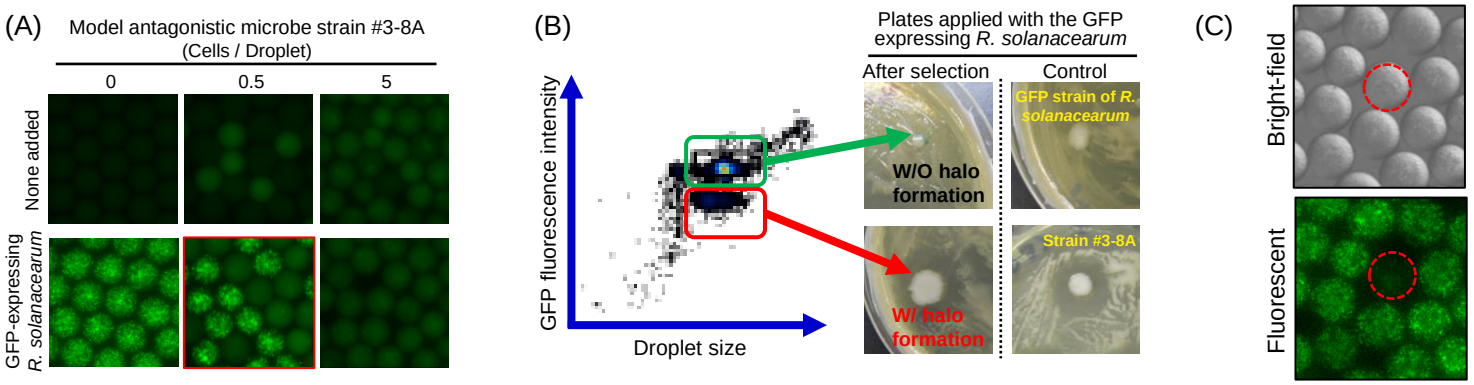

Figure 5-2. Screening of antagonistic microorganisms using GFP-expressing *R. solanacearum* strain. (A) Microscopic images of droplets containing the GFP-expressing pathogen and model antagonist microbial strain #3-8A after incubation, (B) 2D plots of 0.5 cells/droplet samples analyzed with the On-chip Droplet Selector and photographs of dual culture after selection of each population, and (C) microscopic images after incubation of droplets containing the GFP-expressing *R. solanacearum* and soil microorganisms.

Experiments using a GFP-expressing plant pathogen, *Ralstonia solanacearum*, and its model antagonist, strain #3-8A, revealed a correlation between the fluorescence intensity of the droplets and antagonism (Fig. 5-2A and B). When the GFP pathogen and soil microbes were co-encapsulated in droplets and cultured, a significant decay of GFP fluorescence intensity was observed in some droplets, suggesting that the detection and isolation of antagonistic microorganisms is possible (Fig. 5-2C). Compared to conventional methods, this technology, which enables rapid and large-scale co-culture experiments, can screen not only antagonistic microorganisms but also various effective microorganisms, and is expected to become an innovative method in biomanufacturing.

Title of invention: Method for screening effective microorganisms, Appl. No. (Int.): PCT/JP2023/16681 Appl. Date: April 27, 2023

06 Two-dimensional screening for simultaneous evaluation of microbial growth activity and substance production

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The self-sufficiency rate of fats and oils in Japan is low, and a stable supply source is required. *Lipomyces starkeyi*, an oleaginous yeast, is known to produce high levels of lipids. However, in order to use this microorganism as a stable supply source, it is necessary to screen mutant strains with high lipid production capacity. The most common method for screening strains with high lipid production is to evaluate cell size and the amount of lipids accumulated in each cell using a cell sorter, but cell death due to damage during sorting is a problem with this method. Prof. Ogasawara and his team not only succeeded in recovering sorted strains alive, but also established an innovative method to simultaneously evaluate growth and lipid-producing capacity upon sorting.

First, the oleaginous yeast *L. starkeyi* was encapsulated in GMDs and cultured. The team observed that the oleaginous yeast grew well in individual GMDs, and by adding BODIPY dye, which selectively stains lipids, they were able to detect fluorescence of lipids produced in the yeast after incubation (Fig. 6-1A). Next, GMDs cultured with *L. starkeyi* mutant strains were analyzed using On-chip Sort to simultaneously evaluate growth and lipid production capacity, and GMDs containing strains with slow growth and high oil production capacity were selectively recovered (Fig. 6-1B). Finally, the strains obtained from the sorted GMDs were evaluated for growth and lipid production on plates. The results showed that the strains had the same high lipid-producing capacity as the wild-type strain even though the growth rate was one-tenth of that of the latter (Fig. 6-1C). The outcome indicates that this screening method would be useful for obtaining strains for industrial applications.

This method, which allows simultaneous evaluation of growth and lipid production, is expected to be applied to the screening of various microorganisms used in bioprocessing and will not be limited to the screening of oleaginous yeast.

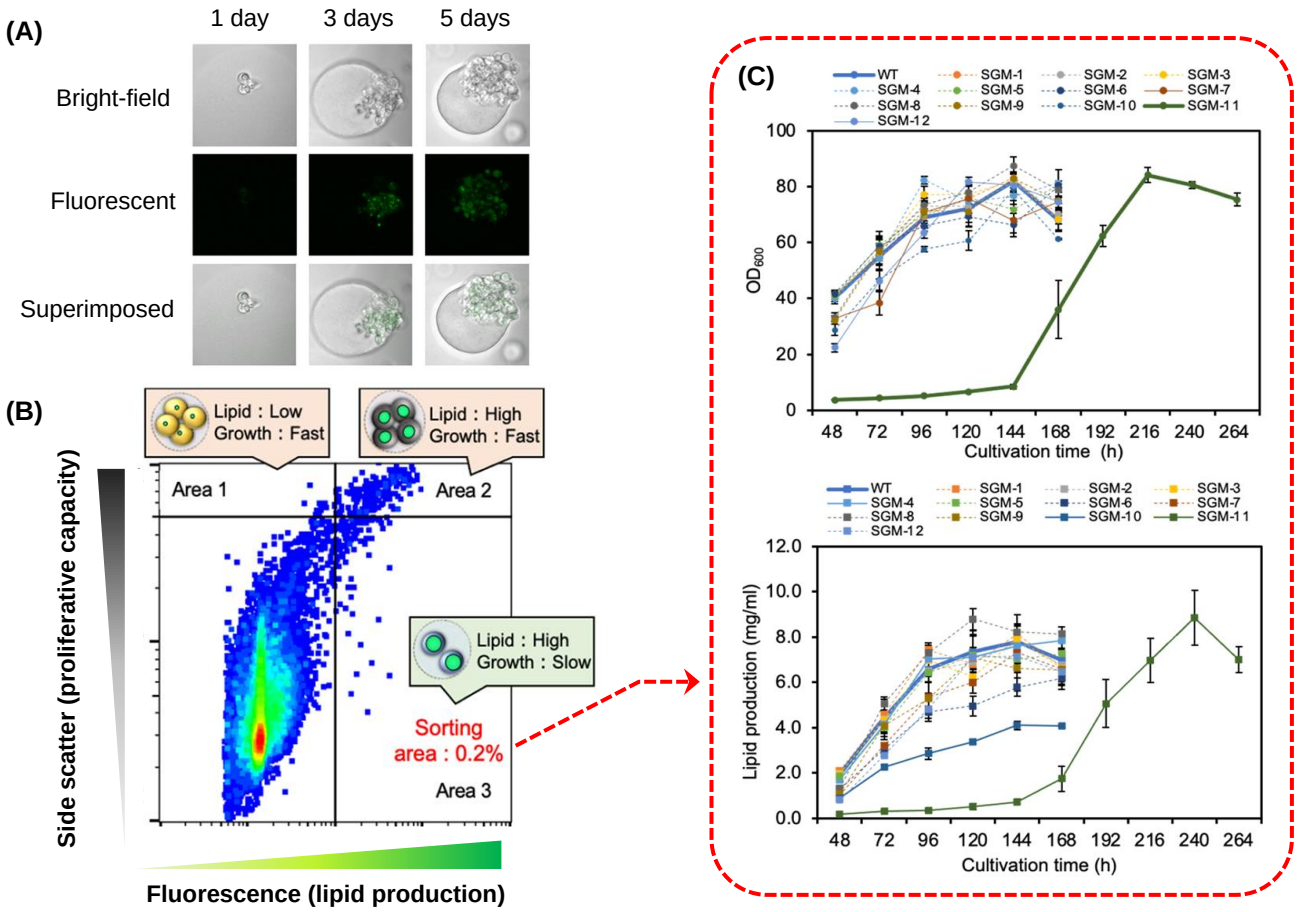

Figure 6-1. Screening of oleaginous yeast using GMDs. (A) Microscopic observation of GMDs containing cultured oleaginous yeast, (B) analysis of GMDs and sorting gate setting using growth and lipid-producing capacity of oleaginous yeast as indices, and (C) evaluation of growth and lipid-producing capacity of strains recovered from sorted GMDs.

Reference

Using gel microdroplets to develop a simple high-throughput screening platform for oleaginous microorganisms
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Journal of Biotechnology **2022** *358*, 46–54.
DOI: 10.1016/j.jbiotec.2022.08.016. https://doi.org/10.1016/j.jbiotec.2022.08.016

07 Potential for high-throughput screening of gut bacteria with On-chip Sort

On-chip products operable in anaerobic chambers

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Gut bacteria and their metabolites have been shown to be involved in maintaining physiological homeostasis and disease development by acting not only on the gut, but also on the whole body. This knowledge has been applied to the identification of gut bacteria that produce useful/toxic metabolites and to the transplantation of gut microbiota. However, since most gut bacteria are anaerobes, the construction of an anaerobic environment using an anaerobic chamber is necessary for research, and limited space makes single-cell or large-scale screening difficult for handling a large number of samples.

Encapsulation of bacteria into GMDs with On-chip Droplet Generator allows for large numbers of single-cell cultures in a small amount of space. Bacteria grown in GMDs can be stained with various fluorescent reagents, and each stained GMD can be analyzed and screened with On-chip Sort. Furthermore, On-chip SPiS enables rapid dispensing into plates (Fig. 7-1A to C). All of these devices are small enough to be installed in an anaerobic chamber and are ideal for screening anaerobic bacteria. A series of operations were performed in an anaerobic chamber using *Bifidobacterium longum* as a model of gut bacteria (Fig. 7-2A and B), which confirmed that encapsulation of a single cell per single GMD, culture, fluorescent staining, sorting, and dispensing of sorted samples to plates can be performed even under anaerobic conditions. In addition, microscopic observation of the plates after dispensing confirmed that it is possible to re-culture the bifidobacteria encapsulated in the GMDs.

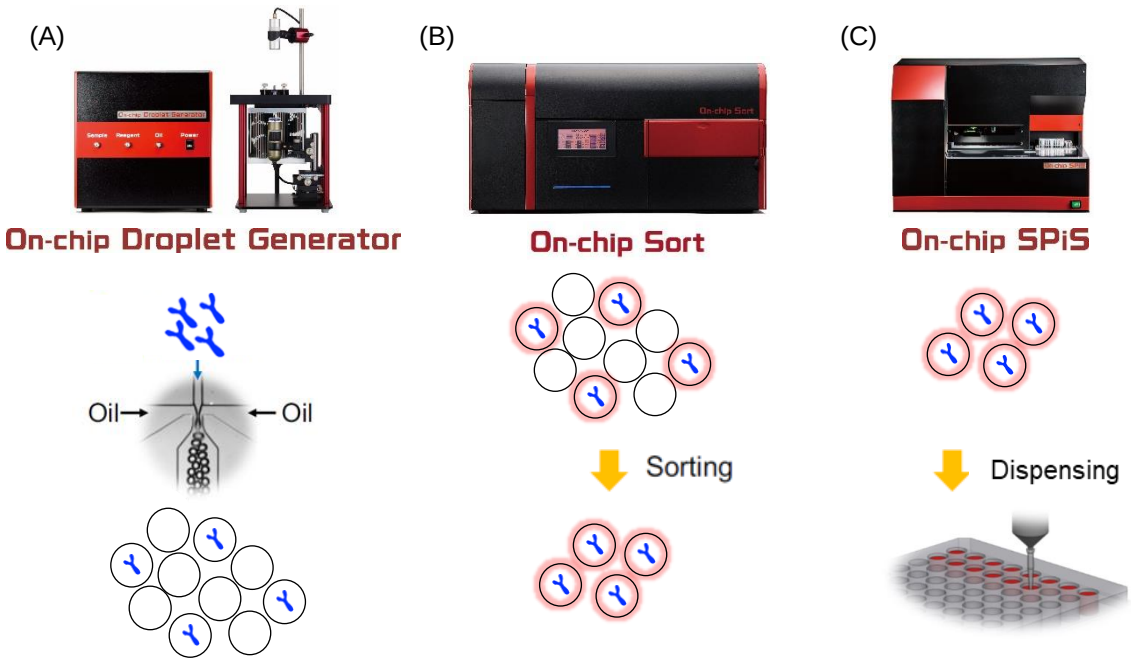

Figure 7-1. Workflow for high-throughput screening of bifidobacteria using GMDs with On-chip Sort. (A) Encapsulation of bifidobacteria into GMDs (a single cell per each one) with On-chip Droplet Generator, (B) fluorescent staining in GMDs and sorting with On-chip Sort, and (C) single cell (GMD) dispensing from sorted GMDs with On-chip SPiS.

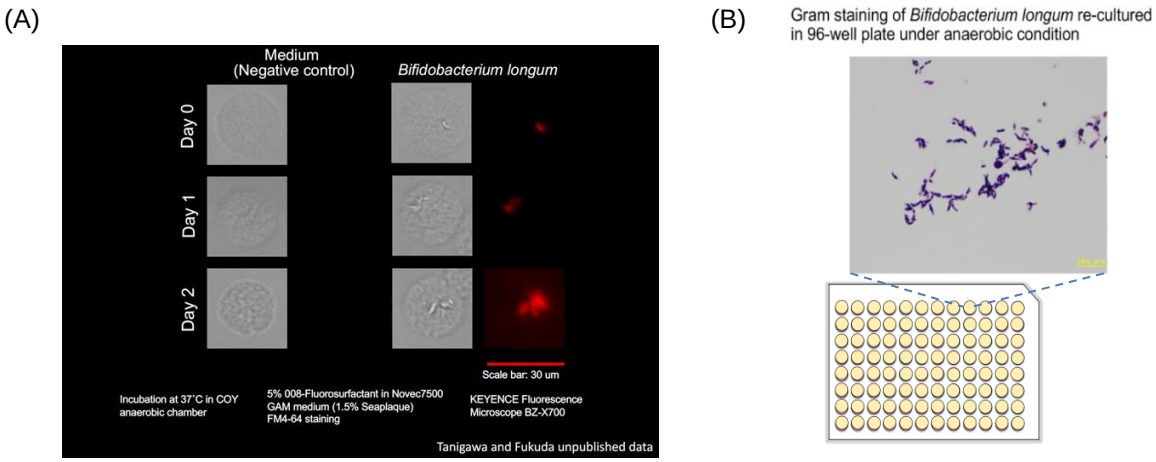

Figure 7-2. Staining properties of *Bifidobacterium longum* after encapsulation and culture in GMDs and re-culturing after sorting and dispensing to plates. (A) Microscopic images on days 0, 1, and 2 after encapsulation in GMDs, and (B) a microscopic image of an FM4-64 stain-positive GMD after being incubated for two days, dispensed into a 96-well plate with On-chip Sort and On-chip SPiS and re-incubated.

08 High-throughput screening for strains with high protein productivity from mutation libraries

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In recent years, the demand for proteins for industrial use, such as useful enzymes and antibodies for biopharmaceuticals, has been increasing. Microorganisms such as yeast and *E. coli*, which can be easily genetically modified and mass cultured, are used to produce such proteins, but microbial screening is essential for producing desired proteins at an industrial level. However, the screening method that combines the commonly used limiting dilution and protein quantification methods is low throughput and time-consuming. In contrast, screening using a cell sorter can be performed at high throughput but using FACS to distinguish cells with high protein productivity remains technically challenging and sorting causes significant damage to cells. Prof. Machida and his colleagues at Kanazawa Institute of Technology have shown that GMDs can be used to detect proteins secreted by microorganisms with fluorescence, and that the microorganisms that produce high levels of proteins can be recovered alive. In this proof of concept study, screening for strains producing high levels of proteins was investigated using budding yeast as a model.

First, a library of mutant strains was prepared by inducing random genetic mutations by UV irradiation in budding yeast, into which plasmids containing a Halo Tag linked downstream of the luciferase gene were transferred. The library was then encapsulated in GMDs and incubated overnight to form colonies. Target proteins secreted from the mutant strains in the GMDs were fluorescently stained by adding the Halo Tag Alexa Fluor 488 Ligand. On-chip Sort was then used to analyze the fluorescence intensity of individual GMDs using the FL2 channel, which can detect emitted light with a wavelength of 488 nm. Three sorting gates were set based on intensity, and the GMDs in each area were sorted (Fig. 8-1A). Observation of the samples obtained from each sorting gate under a microscope indicated that the GMDs in the P7 area did not contain any viable cells but contained dead cells and/or aggregation of fluorescent dye, which generated strong fluorescence (Fig. 8-1B, upper panel). In contrast, a population of cells with strong fluorescence and normal proliferation was observed in the GMDs in the P8 area (Fig. 8-1B, lower panel). In addition, colonies with weaker fluorescence than those in the P8 area were observed in the GMDs in the P9 area (data not shown). Based on these results, the P8 area, where cell populations with strong fluorescence and normal proliferation were observed, was considered an area of cells producing high levels of the target protein, and the GMDs in the P8 area were sorted again. To confirm whether the mutant strains obtained by this method really produced the target protein at high levels, luciferase assays were performed on plates, obtaining P8-7 and P8-12 strains that showed two-to-five times higher levels of protein production than the strain before transformation (Fig. 8-1C).

This method is expected to be used in various biotechnological industries as an efficient screening method for cell strains that produce useful proteins.

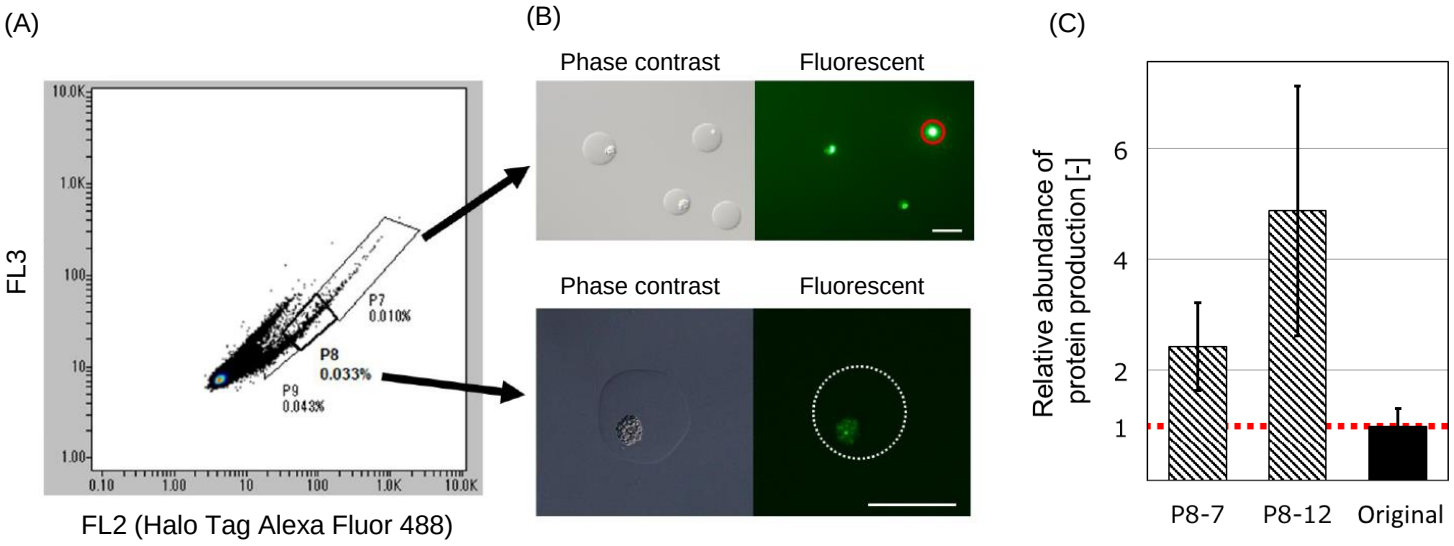

Figure 8-1. GMD-based screening of strains producing high levels of protein. (A) Fluorescence detection of GMDs in which mutant strains were cultured, (B) microscopic images of GMDs in P7 and P8 areas, and (C) protein production of the sorted strains.

Reference

High-throughput screening of high protein producer budding yeast using gel microdrop technology
Fujitani, H., Tsuda, S., Ishii, T., Machida, M.
bioRxiv **2019**
https://doi.org/10.1101/830596

09 Examples of high-throughput single-cell screening of antibody-producing cells using Nanovials

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Cell sorting and single cell analysis using surface marker as indicators are indispensable tools for modern cell biology. However, analysis of cellular functions, such as cytokine and antibody secretion, requires intracellular staining, making cell sorting while maintaining cells in a viable state difficult. Dr. Joseph de Rutte and Prof. Dino Di Carlo's group at the University of California have developed a solution for secretion analysis and cell sorting without the need for intracellular staining that involves culturing single cells inside Nanovials and keeping secreted products inside. This product, Nanovial, is available from Partillion Bioscience Corporation.

Nanovials are microbeads with a spherical inner cavity in the center; they can be modified with various carriers—including antibodies—to hold cells in the cavity. In addition, Nanovials are encapsulated in water-in-oil droplets in an emulsion state and incubated to prevent diffusion of secreted products from cells, and target secreted products are captured by antibodies bound to the cavity. The secreted products that stay in the cavity are stained with fluorescently labeled antibodies to label the target secretions and the cells that secrete them, enabling detection by flow cytometry and sorting. Nanovials are available in sizes ranging from 35 μm to 85 μm, depending on the cell size of interest, including some sizes that existing cell sorters cannot accommodate. The team has shown that On-Chip Sort, a microfluidic cell sorter, can be used to sort a wide variety of cells using secreted products as the indicator (Fig. 9-1A).

For further development of cell therapies as well as biologics such as antibody drugs, single cell analysis and screening based on secreted products is essential. Nanovial, which can be modified in various ways and has broad applicability, can be handled with existing cell sorters and will be a powerful tool to meet these needs.

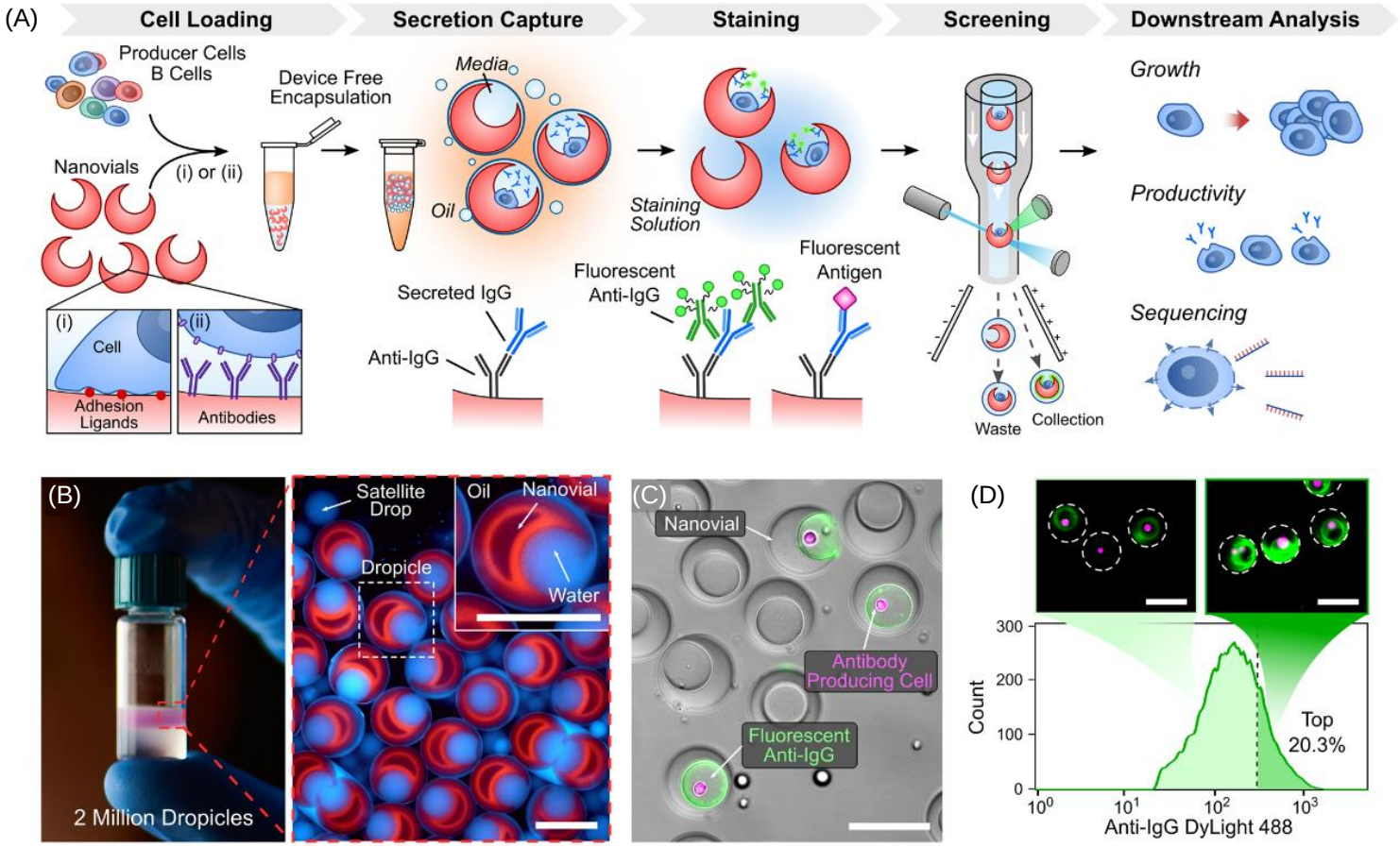

Figure 9-1. High-throughput screening method based on Nanovial using secreted products from cells as an indicator. (A) Workflow of screening antibody-producing cells using Nanovials, (B) Nanovials in water-in-oil emulsion and their enlarged view, (C) microscopic image (fluorescent) of Nanovials incubated in the oil to produce antibodies, followed by oil removal and fluorescence staining, and (D) dot plot from flow cytometry and fluorescent microscopic images of respective fractions after sorting.

Nanovials suspended in a culture medium are easily emulsified by adding oil and pipetting (Fig. 9-1B). Emulsions with cells and medium retained in the cavity can be cultured as they are, and no interference occurs between Nanovials blocked by the oil. In addition, since they can be easily recovered from the oil, subsequent fluorescent staining and flow cytometric analysis are possible (Fig.9-1C and D). In other words, Nanovial is a tool that can function as an ELISA plate that allows flow cytometric analysis and sorting for each well.

Nanovial can be used to screen not only secreted products such as cytokines, but also antigen-specific antibodies and the cells that produce them. In a study using HyHEL-5, a hybridoma that produces anti-egg albumin lysozyme antibodies, it was confirmed that only hybridomas producing the target antibody could be screened by retaining the secreted antibody in the Nanovial cavity with anti-Fc antibody and reacting it with fluorescently labeled egg albumen lysozyme (Fig. 9-2A and B). Furthermore, the team succeeded in sorting HyHEL-5, which was only 0.18% in a population of 1 million cells, including non-targeted anti-Myc-Tag antibody-producing hybridomas, at 90% purity (Fig. 9-2C). It has also been confirmed that the sorted cells can be used for amplification of antibody genes by RT-PCR and for expansion cultures (Fig. 9-2D).

By applying this method, it is possible to sort B cells producing target antibodies from immunized mice. In fact, when B cells from mice immunized with ovalbumin (OVA) were analyzed and sorted using a combination of Nanovial and fluorescent OVA, a variety of positive Nanovials were found, including those with strong signals only on the cell surface and those with no signals on the cell surface but strong signals from the Nanovial.

These data suggesting that Nanovial screening can obtain not only pre-matured B-cell which have been sorted using the existing method based on B-cell receptor staining with Fluorescent-antigen, but that highly differentiated plasma B cells can also be detected/sorted simultaneously (Fig. 9-2E).

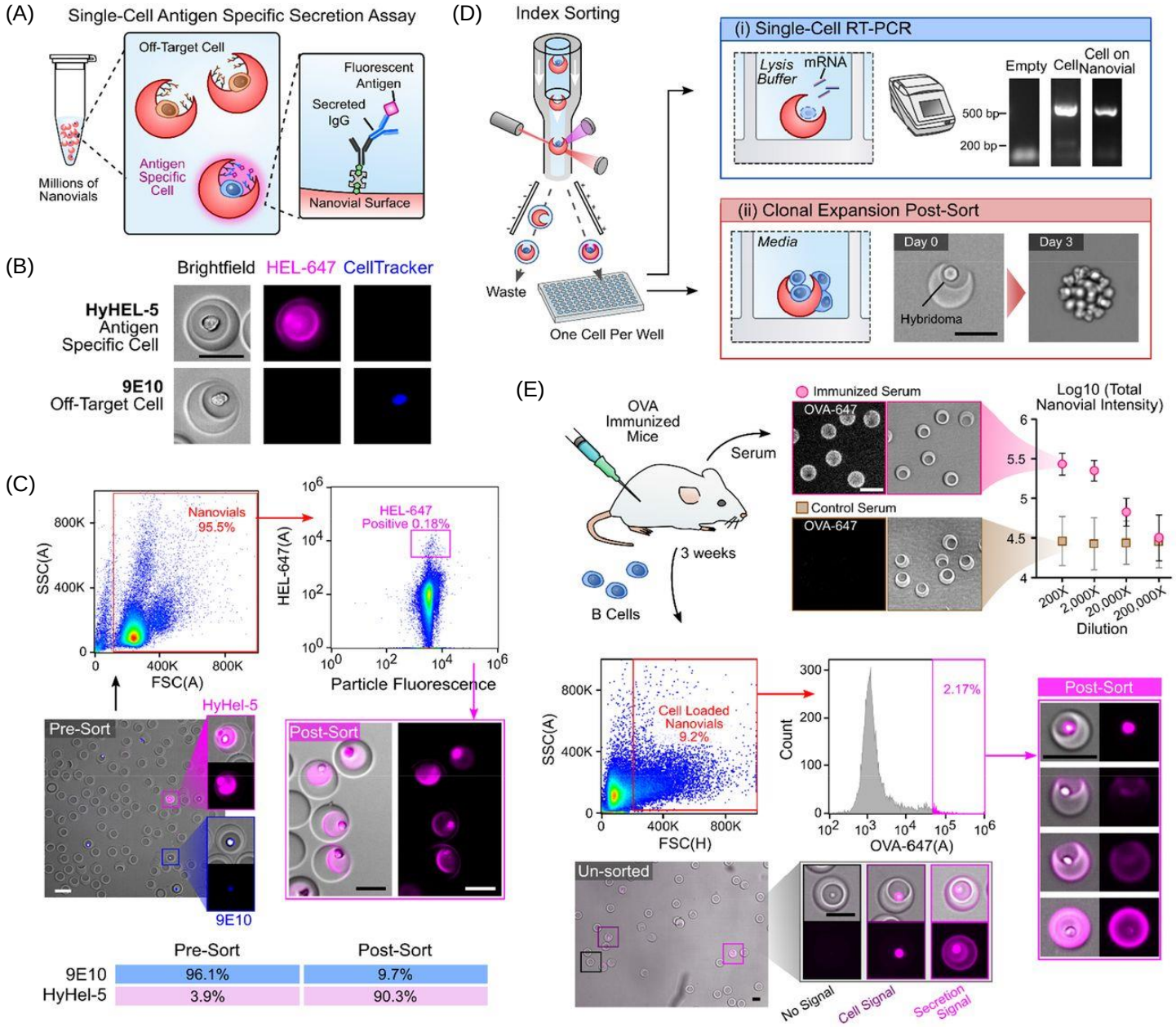

Figure 9-2. Nanovial-based screening of antigen-specific antibody-producing cells. (A) Schematic illustration of antigen-specific antibody-producing cell screening using fluorescent antigens, (B–D) detection of cells producing target antibody using a model system of HyHEL-5 and fluorescently labeled antigen (B–D), analysis and sorting results (C), and an example of amplification of antibody genes by RT-PCR and expansion culture (D), and (E) detection of anti-OVA antibodies in the serum of OVA-immunized mice and screening of anti-OVA-producing B cells.

Reference

Suspendable Hydrogel Nanovials for Massively Parallel Single-Cell Functional Analysis and Sorting
Joseph de Rutte, Dino Di Carlo. *et al.*
ACS Nano **2022**, *16*, (5), 7242–7257.
DOI: 10.1021/acsnano.1c11420. https://doi.org/10.1021/acsnano.1c11420

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