Application Note

Evaluation and high-throughput screening system of microorganisms based on their enzyme activity which is accomplished by ACA a novel hydrophilic fluorescent probe

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Introduction

Water-in-oil droplets (WODLs) are pL-nL scale water droplets dispersed in an oil phase, enabling culturing and single-cell analysis of encapsulated animal cells or microorganisms with minimal interference between samples. Considering WODLs as micro-reaction chambers, they can be used for analyzing cellular metabolic activity and enzymatic reactions over time, which could not be detected by canonical cell surface markers. However, it is difficult to apply to large-scale screening, because most of the fluorescent probes that are commonly used for detecting enzyme activity diffuse to the oil phase due to their hydrophobicity.

To overcome this problem, a novel hydrophilic fluorescent substrate, dipeptidyl 7-aminocoumarin-4-acetic acid (dipeptidyl-ACA), that would not leak from WODLs, was developed as a probe for detecting microbial enzyme activity in WODLs. In this study, WODLs with encapsulated dipeptidyl-ACA and microorganisms were generated by our droplet generator, On-chip Droplet Generator, and the microorganisms inside the WODLs were subsequently screened by our microfluidic chip cell sorter, On-chip Sort, based on dipeptidyl peptidase (DPP) activity. The results of this study indicate that the combination of hydrophilic substrate and WODLs sorting could be a powerful tool for microorganisms screening based on enzyme activity in a high-throughput manner.

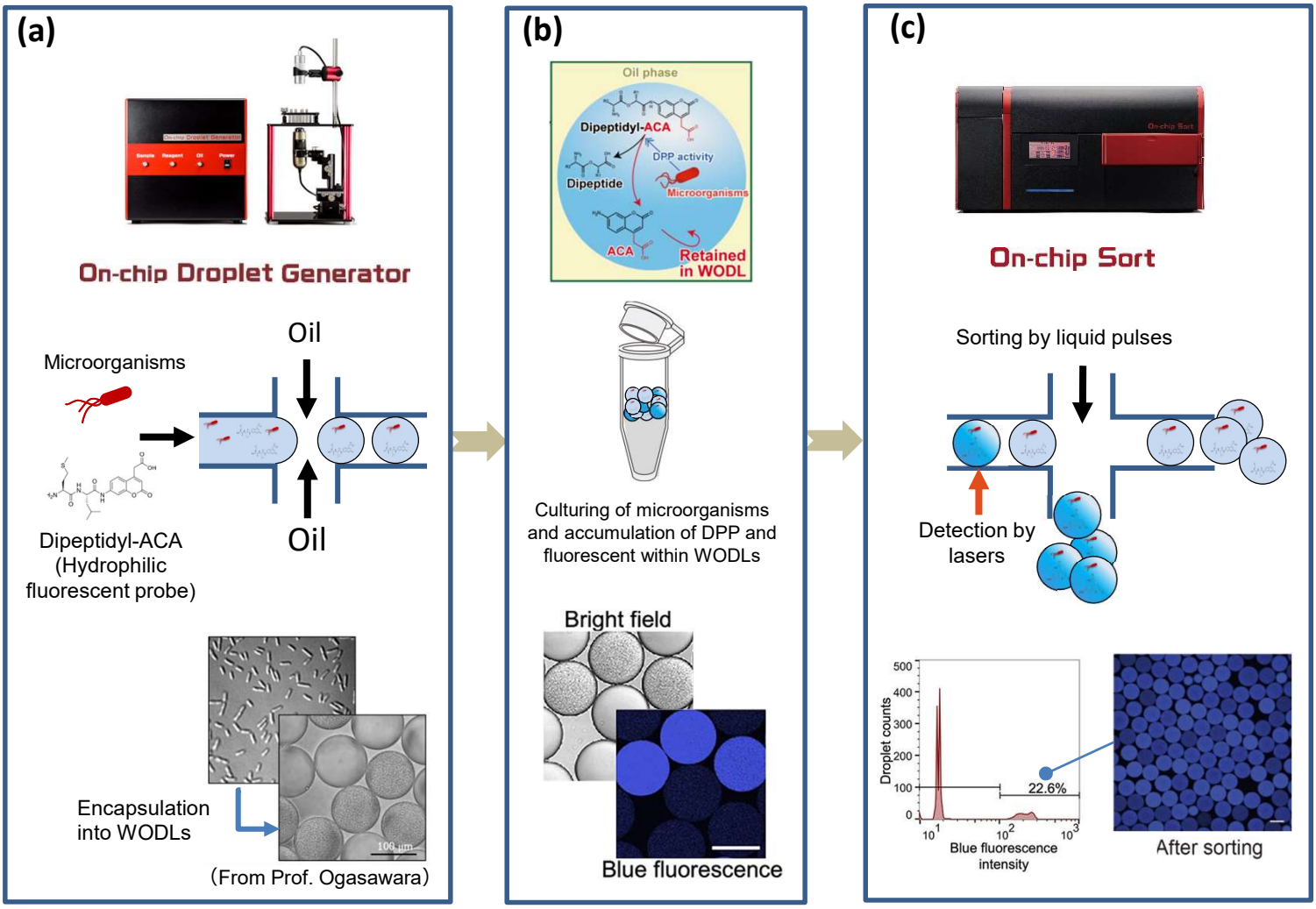

Fig. 1. Experimental workflow of enzyme-producing microorganisms screening by On-chip Droplet Generator, Dipeptidyl-ACA, and On-chip Sort

(a) Dipeptidyl-ACA and microorganisms encapsulating in WODLs by On-chip Droplet Generator. **(b)** Blue fluorescent signal in WODLs as a result of ACA hydrolyzed by bacterial derived DPP. **(c)** Detection and sorting of high enzyme-producing microorganisms by dipeptidyl-ACA and On-chip Sort.

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Background and Results

Exploration of hydrophilic substrates for the detection of enzyme activity in WODLs

7-Amino-4-methylcoumarin (AMC) is an amino group-linked derivative of coumarin at position 7 and emits strong blue fluorescence. The amino group in AMC works as a reactive group that can form an amide bond between AMC and various peptides, and this linkage causes a temporary decrease in its fluorescence. However, AMC released by hydrolysis or enzymatic reaction of the amide bond again emits strong fluorescence, and thus, AMCs conjugated with arbitrary peptides are widely used as fluorescent substrates to detect various enzyme activities.
On the other hand, AMC is not suitable for detecting enzyme activity in WODLs due to its hydrophobicity and diffusibility to the oil phase. To overcome this problem, a novel hydrophilic AMC-derived fluorescent probe, dipeptidyl-ACA, was synthesized.

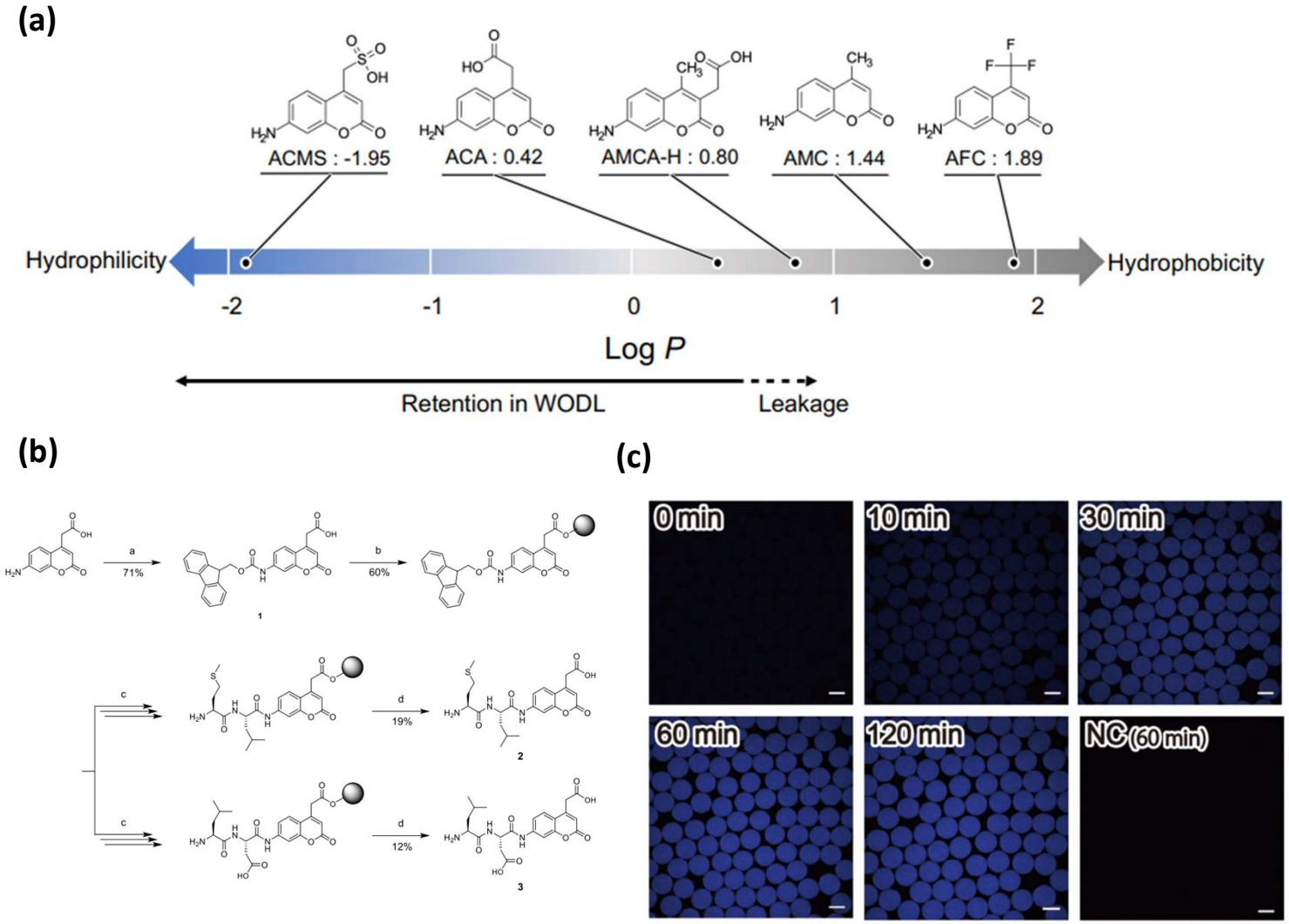


Fig. 2. Comparison of hydrophobicity between ACA and other fluorescent probes, and assessment of dipeptidyl-ACA as fluorescent peptide substrate.
(a) Relationship between hydrophobicity and retention properties of coumarin derived-probes in WODLs. **(b)** Synthesis steps of ACA-conjugated peptides (Met-Leu-ACA, Leu-Asp-ACA). **(c)** Confocal micrographs of fluorescing WODLs brought about by Met-Leu-ACA and purified DPP reaction.

At first, the relationship between hydrophobicity and retention of the ACA, AMC, and other fluorescent probes was confirmed. As a result, ACA showed lower hydrophobicity than AMC(Fig 2a), a general fluorescent probe, and retaining in WODLs for 168 hours. On the other hand, although ACMS showed lower hydrophobicity than AMC, inhibits the enzyme reactions. Thus, we concluded that ACA is a more promising candidate and conjugated it with 2 peptides for further investigation (Fig 2b). In fact, under confocal microscopy, DPP activity could be detected (represented by the blue fluorescence) in WODLs containing dipeptidyl-ACA and purified DPP (Fig 2c).

Assessment of dipeptidyl-ACA, a novel hydrophilic fluorescent probe, with model microorganisms

To investigate whether the synthesized dipeptidyl-ACA emits the fluorescence by the microorganisms-derived enzyme and whether it is possible to applying to screening practically or not, we assessed these three following tests with *Pseudoxanthomonas mexicana* WO24, a DPP-producing bacteria.

(a) Whether the dipeptidyl-ACA can detect enzyme activity as fluorescent, and isolate it?
(b) Whether the dipeptidyl-ACA can selectively isolate microorganisms which produce target enzymes from various microorganism pools.
(c) Whether the dipeptidyl-ACA can be screened precisely, in the case of parallel assessment that is performed with other fluorescent probes.

The DPP-producing model microorganisms *P. mexicana* WO24 or non-producing, RFP-expressing microorganisms *Escherichia coli* DH5α were encapsulated with dipeptidyl-ACA in WODLs using On-chip Droplet Generator, and the fluorescent signals after 24 hours of incubation were analyzed using On-chip Sort. In Fig 3c, the accumulation of RNase accompanying proliferation was detected as green fluorescence using FNAP (see QR code below), which has been sold by our company, and the proliferation potential of the microorganism is evaluated at the same time.

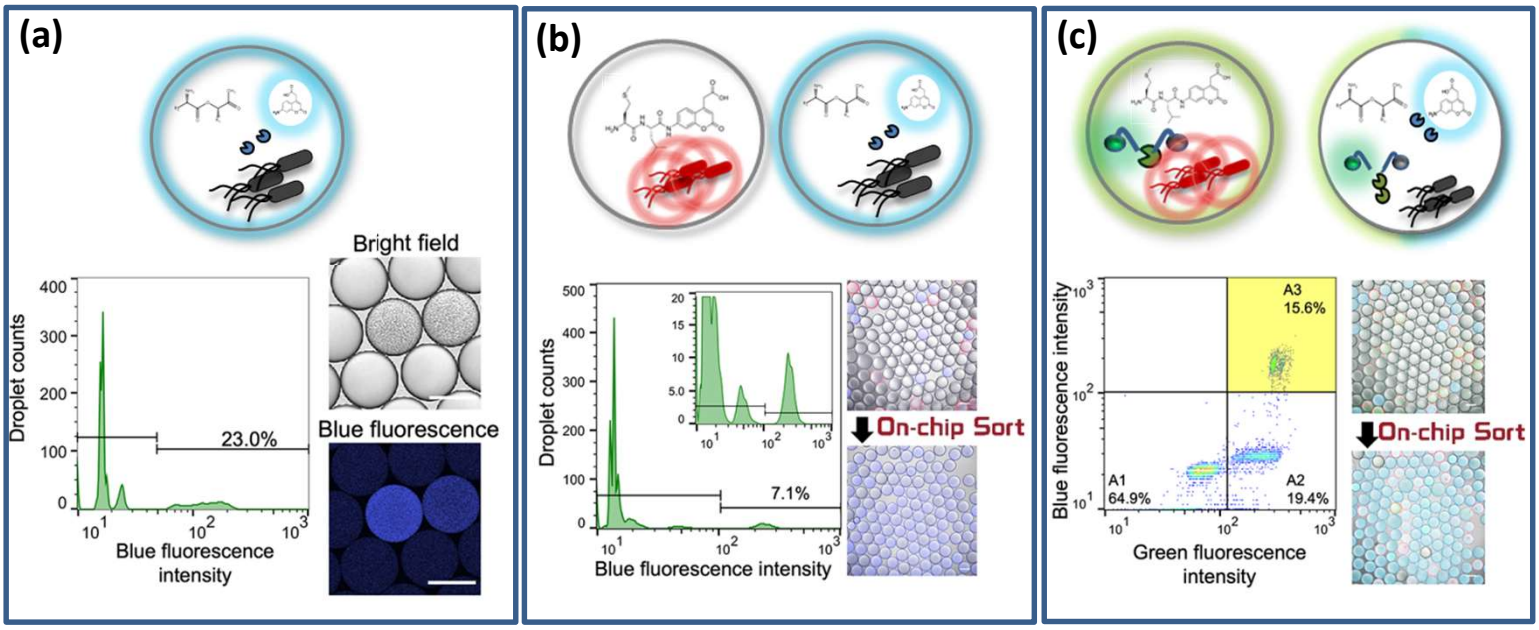


Fig. 3. Dipeptidyl-ACA for detection of enzyme-producing microorganisms and establishment of the screening system.
(a) Detection of microorganisms-derived dipeptidase activity by dipeptidyl-ACA. **(b)** Detection and sorting dipeptidase producing microorganisms as a blue fluorescing WODLs by On-chip Sort. **(c)** Screening of dipeptidase high-producing and high-growth microbes by combination of dipeptidyl-ACA and FNAP with On-chip Sort.

Dipeptidyl-ACA enabled the detection of DPP activity produced by *P. mexicana* WO24 in WODLs as blue fluorescence (Fig 3a). Furthermore, by sorting with On-chip Sort, it was possible to selectively screen the WODLs showing dipeptidase activity (Fig 3b) and microorganisms with high growth potential by FNAP from a mixture of red fluorescent microorganisms (Fig 3c). Thus, dipeptidyl-ACA is capable of detecting bacteria-derived enzyme activity, even though the presence of other fluorescent proves such as FNAP reagents. These data indicate that dipeptidyl-ACA can be used in conjunction with a variety of applications.

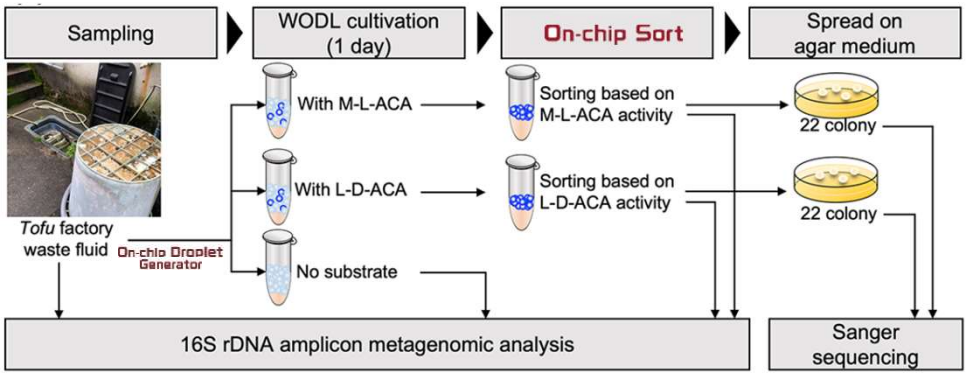
WODLs screening with fluorescent-nucleic acid probe (FNAP-sort)

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, et al. (2019) PLOS ONE 14(4): e0214533. https://doi.org/10.1371/journal.pone.0214533

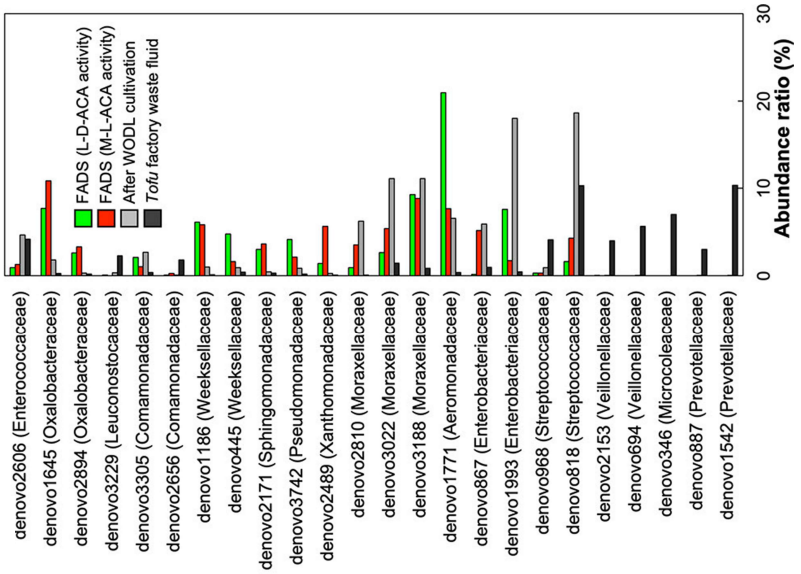
Illustration of DPP-producing microorganisms screening with Dipeptidyl-ACA from the environmental sample

To investigate whether the dipeptidyl-ACA can screen environmental samples based on their DPP activity, we chose the tofu factory waste fluids that are expected to be abundant in peptides.

(a)



(b)



(c)

Sample specificity	No.	Determined strains using BLAST: Strains	Identify (%)
Common	1	*Sphingomonas yabuuchiae* strain A1-18	99.7
	2	*Pseudoduganella danionis* strain E3/2	98.2
	3	*Stenotrophomonas rhizophila* strain e-p10	99.0
	4	*Chryseobacterium camelliae* strain THG C4-1	95.2
	5	*Flavobacterium chilense* strain LM-09-Fp	99.6
	6	*Aeromonas hydrophila* strain ATCC 7966	99.7
M-L-ACA specific	7	*Enterobacter soli* ATCC BAA-2102 strain LF7	99.4
	8	*Falsarthrobacter* (*Arthrobacter*) *nasiphocae* strain M597/99/10	98.1
	9	*Janthinobacterium agaricidamnosum* strain NBRC 102515	99.4
	10	*Stenotrophomonas terrae* strain R-32768	99.0
	11	*Lactococcus lactis* strain NBRC 100933	99.8
	12	*Pantoea agglomerans* strain DSM 3493	99.2
	13	*Klebsiella grimontii* strain SB73	99.0
	14	*Cronobacter dublinensis* subsp. *lausannensis* strain E515	99.6
	15	*Moraxella osloensis* strain DSM 6998	99.1
	16	*Pseudomonas otitidis* strain MCC10330	99.8
	17	*Brevundimonas faecalis* strain CS20.3	99.1
	18	*Massilia violacea* strain CAVIO	99.8
L-D-ACA specific	19	*Aeromonas allosaccharophila* strain CECT 4199	99.7
	20	*Pseudomonas protegens* strain CHA0	99.8
	21	*Chryseobacterium gallinarum* strain 100	99.5
	22	*Paenarthrobacter nitroguajacolicus* strain G2-1	99.8
	23	*Chryseobacterium taihuense* strain THMBM1	97.6
	24	*Flavobacterium ginsengiterrae* strain DCY55	96.4
	25	*Pararheinheimera arenilitoris* strain J-MS1	98.1
	26	*Chryseobacterium arachidis* strain 91A-593	98.5
	27	*Sphingomonas trueperi* strain NBRC 100456	99.9
	28	*Pseudomonas japonica* NBRC 103040	98.9
	29	*Cloacibacterium haliotis* strain WB5	98.6
	30	*Brevundimonas terrae* strain KSL-145	99.4

Fig 4. Illustration and results of high-throughput screening with ACA and FNAP-sort.
(a) Workflow of high-throughput screening based on DPP-activity. **(b)** The abundance ratio of each OTUs which is determined by 16s rRNA gene sequencing in pre- and post-screening samples (Green bar; Leu-Asp-ACA, Red bar; Met-Leu-ACA. **(c)** The list of obtained microorganisms that are identified by 16S rRNA sequencing and BLAST.

After WODLs sorting by fluorescent of dipeptidyl-ACA as an index with On-chip Sort (Fig 4a), the increased abundance ratio of some specific phylum which is known as DPP-producing microorganisms had confirmed (Fig 4b). In addition, 16S rRNA gene sequencing and BLAST revealed that almost all the obtained colonies after sorting carry the DPP genes (Fig 4c). These data indicate that WODLs sorting carried out by dipeptidyl-ACA and On-chip Sort is a promising screening system for even in environmental samples.

Previously, it is difficult to detect the enzyme activity in WODLs due to almost fluorescent probes diffused into the oil phase. ACA, a novel hydrophilic fluoresce probe overcomes this hurdle and combination with On-chip Sort, the first (and only one available for commercial use) machine in the world capable of WODLs sorting, enables activity-based microorganisms screening. Furthermore, ACA can be bound to various peptides, which will surely expand and accelerate explore of effective microorganisms.

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