Positive dielectrophoresis–based Raman-activated droplet sorting for culture-free and label-free screening of enzyme function in vivo

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The potential of Raman-activated cell sorting (RACS) is inherently limited by conflicting demands for signal quality and sorting throughput. Here, we present positive dielectrophoresis–based Raman-activated droplet sorting (pDEP-RADS), where a periodical pDEP force was exerted to trap fast-moving cells, followed by simultaneous microdroplet encapsulation and sorting. Screening of yeasts for triacylglycerol (TAG) content demonstrated near-theoretical-limit accuracy, ~120 cells min⁻¹ throughput and full-viability preservation, while sorting fatty acid degree of unsaturation (FA-DU) featured ~82% accuracy at ~40 cells min⁻¹. From a yeast library expressing algal diacylglycerol acyltransferases (DGATs), a pDEP-RADS run revealed all reported TAG-synthetic variants and distinguished FA-DUs of enzyme products. Furthermore, two previously unknown DGATs producing low levels of monounsaturated fatty acid–rich TAG were discovered. This first demonstration of RACS for enzyme discovery represents hundred-fold saving in time consumables and labor versus culture-based approaches. The ability to automatically flow-sort resonance Raman–independent phenotypes greatly expands RACS’ application.

INTRODUCTION

A single cell is the basic unit of function for life on Earth. A single-cell Raman spectrum (SCRS) consists of thousands of resonance or nonresonance Raman peaks, which individually or in combination can model a particular biochemical or metabolic phenotype of the cell; thus, SCRS can capture the metabolic state of a cell just like a function-based instant photo (1). By sorting cells via SCRS, Raman-activated cell sorting (RACS) holds great promise as a powerful platform for screening biological parts, modules, or classes, as it is label-free, culture-free, noninvasive, landscape-like, and broadly applicable to all kinds of cells (1–3). However, the potential of RACS is inherently limited by the conflicting demands for SCRS acquisition time: longer for higher sensitivity to nonresonance Raman peaks yet shorter for higher throughput and cell viability preservation. Specifically, among the present members of the RACS family, Raman tweezers (4, 5) and Raman-activated cell ejection (RACE) (6–8), which acquire SCRS and then sort largely static cells, can detect nonresonance Raman peaks (which are much weaker than resonance Raman peaks) yet suffer from relatively low throughput. For example, an automated flow mode Raman tweezer accomplished 3.3 to 8.3 cells min⁻¹ in sorting metabolically active bacterial cells (based on the C-D band that is a nonresonance Raman peak) from mouse colon microbiota (9); however, the inability of optical tweezers to quickly trap and move a fast-moving cell, due to its weak force, has hindered further throughput increase. On the other hand, Raman-activated microfluidic sorting (RAMS) (10, 11) and Raman-activated droplet sorting (RADS) (12), which use a trap-free or trapping-and-release strategy via positive dielectrophoresis (pDEP) instead of Raman tweezers, can sort at hundreds of cells per minute (12). However, the type and scope of phenotypes sortable by RAMS and RADS are limited to only those associated with resonance Raman peaks (whose signals are much stronger than nonresonance peaks, associated with only a few classes of cellular compounds such as pigments), because of their trap-free strategy or use of PDMS (which is of high Raman background) as the structural material. Consequently, a RACS device featuring both generality in tackleable phenotypes and high-throughput sorting is in urgent need (Fig. 1A) (1).

To address this challenge, here, we introduce pDEP-based RADS (pDEP-RADS), which combines the strength of Raman tweezers and RADS and is capable of sorting microbial cells via nonresonance Raman pattern of SCRS at hundreds of cells per minute (Fig. 1B). Sorting of yeasts via pDEP-RADS for triacylglycerol (TAG) content demonstrated high enrichment ratio at a near-theoretical limit, a throughput of ~120 cells min⁻¹, and 100% preservation of vitality, while sorting for fatty acid degree of unsaturation (FA-DU) reported ~40 cells min⁻¹ throughput and ~82% accuracy. From a yeast library expressing algal diacylglycerol acyltransferases (DGATs), pDEP-RADS readily identified two previously unknown enzyme variants that exhibit weak activity synthesizing monounsaturated FA (MUFA)–rich TAG, which are undetectable by fluorescence-activated cell sorting (FACS). Such culture-free, label-free, and noninvasive sorting of enzyme activity in vivo can save time, consumables, and labor by one to two orders of magnitude, as compared to conventional approaches. Moreover, the ability to sort microbial cells via nonresonance Raman pattern at hundreds of cells per minute greatly expands the reach of RACS in mining enzymes or cells from nature.

RESULTS

Design of the pDEP-RADS system

Development of a RACS with high signal sensitivity (i.e., to nonresonance peaks) and high throughput requires the following: (i) fast-moving
single cells must be trapped and aligned with the laser spot precisely; (ii) SCRS must be acquired efficiently; and (iii) target cells must be sorted automatically with high accuracy. To achieve these goals, the pDEP-RADS system mainly consists of a Raman microspectroscopy for acquiring and analyzing the SCRS of loaded cells, a microfluidic chip equipped with pumps for controlling buffer flow, a function generator to generate pDEP to trap single cells, and a high-voltage amplifier to sort droplet that harbors target cells (Fig. 1C). To avoid the interference of Raman background from the chip, we fabricated its parts with quartz (few Raman peaks) as a substrate (Fig. 1D).

**Precise trapping and alignment of fast-moving single cells with laser spot via pDEP**

The pDEP-RADS procedure, which is integrated in the chip and synchronized by multithreading workflow via QSpec (Materials and Methods and fig. S1), includes cell loading and focusing, pDEP-based single-cell trap and release, SCRS acquisition, droplet encapsulation of a cell, and DEP-based droplet sorting for either nontarget or target cells (Fig. 2A). Key for the procedure to acquire SCRS of quality to discriminate nonresonance Raman peaks yet without sacrificing throughput is the trapping of fast-moving single cells at the laser spot for a sufficient period for Raman exposure, via pDEP ([11](#)) in a quartz chip [which is much stronger than optical tweezers ([9](#))].

To generate the pDEP field, we designed an electrode array rather than a single pair of electrodes to trap and deliver the fast-moving single cells step by step to the laser spot for SCRS acquisition (fig. S2A and movie S1). For structural material of the electrode array, indium tin oxide (ITO) rather than metals (e.g., gold, silver, and copper, which cause photothermal damage under the 532-nm Raman laser) was chosen so as to facilitate downstream high-quality SCRS acquisition (fig. S3, B and C). To precisely align the trapped single cells
Efficient acquisition of SCRS in pDEP-RADS

To improve the sensitivity of SCRS, we used quartz (high light transmittance and low Raman background) as the chip substrate. Moreover, the electrode array–based pDEP delivers single cells to the laser spot sequentially, also facilitating efficient SCRS acquisition. Furthermore, the SCRS processing time was reduced by directly reading out from the electron-multiplying charge-coupled device (EMCCD) and optimizing the acquisition, trigger, and readout modes of EMCCD.

To evaluate system stability in the Raman acquisition, we analyzed identical polystyrene (PS) beads of 10 μm in diameter via our pDEP-RADS device (Materials and Methods). Among the series of raw Raman spectra from more than 100 beads (fig. S3A), the 1001 cm\(^{-1}\) band, which is among the most prominent exhibits an SD of ±4.45% in intensity (fig. S3B). This suggests a high degree of signal reproducibility that contributes to system stability in acquiring the SCRS (fig. S3C and movie S5). As a result, the acquisition time for detecting TAG signal (based on nonresonance peaks) in a yeast SCRS was averaged with laser spot, we designed the electrode array as angled (fig. S2D) rather than flatted as previously used (11), which focuses the strongest pDEP field at the angles. In this way, the single cells are dragged to the angles upon the repeated trapping and release (fig. S2E and movie S2) and precisely immobilized at the angle of last electrode (aligned to the laser spot; fig. S2A). Furthermore, by adjusting pDEP frequency, the trapping duration can be tuned to accommodate a wide range of phenotypes (since each may feature a distinct optimal SCRS acquisition time (fig. 2B, fig. S2F, and movie S3). Notably, to prevent adhesion of cells to the electrodes (especially at the laser spot), cell suspensions were supplemented with 0.1% Tween 20 nonionic detergent. With such design, the highest loading of the chip was optimized as 6 cells μl \(^{-1}\) (3.6 μl hour \(^{-1}\) for sample and 2.4 μl hour \(^{-1}\) for focusing buffer; fig. S2, G and H), i.e., 3.7 mm s \(^{-1}\) in the channel. By applying an alternating current (ac) of 16 Vp-p (voltage peak-peak) at 10 MHz, efficiency of both trapping and alignment reached >95% (fig. 2C, fig. S2, and movie S4), setting the stage for efficient SCRS acquisition.
reduced to 50 ms (Fig. 2D), which is sufficient since percentage of detectable target cells did not increase despite the intensification of SCRS along with acquisition time extension (Fig. 2E and fig. S3, D to G).

Notably, in pDEP-RADS, SCRS was acquired continuously; thus, matching acquisition time and trapping duration precisely is a challenge. To ensure that each cell undergoes a complete Raman exposure period, we set the trapping duration as doubled the acquisition time and then the target cells released immediately by triggering an interruption on pDEP via a relay (once the SCRS meets the quality criteria).

**DEP-based simultaneous droplet encapsulation and sorting of cells**

To achieve rapid sorting while maintaining a stable cell stream, we used DEP-based sorting of droplets that each encapsulate a target cell (Fig. 2, F and G, and movie S6). To avoid the interference to SCRS acquisition from the oil phase or from the lensing effect of convex/concave shape of droplet surface, we proposed to interrogate SCRS before droplet encapsulation. Accordingly, the single-cell droplet encapsulation unit was placed after the SCRS acquisition unit in the chip (Fig. 1D). Further downstream is the droplet sorting unit for simultaneous encapsulation and sorting, which increases sorting accuracy and simplifies system design. On the basis of the cell loading velocity of 6 μl hour⁻¹ (3.6 μl hour⁻¹ for sample and 2.4 μl hour⁻¹ for focusing buffer; fig. S2, G and H), the optimal flow rate for oil was 180 μl hour⁻¹, which generated 50-μm-diameter droplets. Samples of ~7.63 × 10⁶ cells ml⁻¹ and ~2.50 × 10⁶ cells ml⁻¹ were loaded (Supplementary Materials and Methods). A 15 ms of 600–Vp-p pulse voltage was applied to sort the target droplets.

**Performance of pDEP-RADS in sorting TAG-synthetic activity**

TAG is a potential source of biofuels, food, and nutrients (13). Onto each TAG backbone are three FA moieties that can be saturated FA (SFA), MUFA, or polyunsaturated FA (PUFA). The DU of these FAs determines the application area, economical value, and market potential of oil products, e.g., either as transportation fuel or nutrients. Therefore, discovering potent and substrate-specific DGATs that assemble TAGs of various DU from diacylglycerol and a particular FA has been of keen interest (14). Notably, SCRS can quantify intra-cellular levels of TAG (based on a Raman peak of 2867 cm⁻¹) (15) and its DU (via the ratio of the C=H stretch and the CH₂ bend) (16). Hence, we started by testing pDEP-RADS in screening the in vivo activity of DGATs.

For this purpose, exogenous DGAT genes were expressed in Saccharomyces cerevisiae strain H1246 (a TAG-deficient quadruple knockout mutant that harbors knockouts of DGA1, LRO1, ARE1, and ARE2), which then underwent a 3-day induction phase for TAG production (Materials and Methods) (17). The TAG (+) ScDGA1 cells (expressing DGA1, which was validated as a TAG-synthetic DGAT from S. cerevisiae SCY062) (17) serves as a positive control, while TAG (−) cells (transformed with empty vector) serves as a negative control (fig. S4, A and B). Thin-layer chromatography (TLC) analysis of bulk cells revealed that TAG was undetectable in TAG (−) cells, while a prominent spot corresponding to TAG appeared in ScDGA1 cells (Fig. 3A), which, per gas chromatography–mass spectrometry (GC-MS), accumulated a considerable level of TAG (18.51% per total lipid versus 0.15% for negative-control cells).

SCRS of ScDGA1 cells revealed a prominent peak of 2867 cm⁻¹ (assigned as C–H₂ and C–H₃ asymmetric and symmetric stretches), which was shown to positively correlate with TAG content [R² (coefficient of determination) = 0.89; Fig. 3B] (15). Intensity of 2867 cm⁻¹ (defined as I₂867 - I₂879) of all the negative-control cells (>100 cells) were <0, while that of >60% of ScDGA1 cells were >0 (Fig. 3C). Hence, “I₂867 - I₂879 > 0” can serve to sort TAG-producing cells. Furthermore, to confirm that “I₂867 - I₂879 > 0” was caused by ScDGA1 cells rather than background noise, an intensity of 2950 cm⁻¹ (assigned as C–H stretching) was considered. Under 50-ms acquisition time, the presence of a cell in laser point always corresponded to I₂950 - I₁800 > 150 (1800 cm⁻¹ was used here because of the absence of biological molecules vibrations from 1800 to 1840 cm⁻¹). Therefore, to screen for TAG-producing cells, the sorting criteria was set as “I₂867 - I₂879 > 0 and I₂950 - I₁800 > 150.”

To probe the sorting efficiency, TAG-producing and non–TAG-producing cells after induction were 1:1 mixed and underwent the pDEP-RADS. Among the more than 60 randomly selected post–pDEP-RADS cells verified for TAG content, only 3 failed to meet the preset sorting criterion (fig. S4C). On average, purity of TAG-producing cells was elevated to ~96% (with “Waste” containing only less than 10% target cells; Fig. 3D), suggesting high-sorting efficiency. To test whether and to what degree the pDEP-RADS process affects vitality of cells, we conducted plate-based culture, which revealed no difference in live-cell concentration between unsorted and sorted cells, suggesting full preservation of cell vitality by pDEP-RADS (Fig. 3E).

To further validate the device, we mixed the two types of yeast cells with a series of ratios and underwent pDEP-RADS. At low dilutions (target versus nontarget of 1:2 to 1:10⁴), high sorting accuracy was achieved (Fig. 3, F and H), reaching a theoretical enrichment maximum (decided by the extent of sample dilution, i.e., the initial concentration; Fig. 3H). The sorting purity reached 83.33%, which corresponds to an enrichment ratio of ~830 (sorted concentration / initial concentration) within one screening run, even at a high dilution (initial concentration of 0.1%). Thus, the pDEP-RADS system is capable of specifically selecting rare events. Under 1:10 dilution, among the 20 random post–pDEP-RADS clones, 18 expressed the target gene of ScDGA1 (Fig. 3G), supporting a sorting accuracy of >90%. Therefore, pDEP-RADS is robust in screening enzymes (and host cells) for the TAG level.

**Performance of pDEP-RADS in sorting synthetic activity of PUFA/MUFA-containing TAG**

To test whether pDEP-RADS can sort via DU those enzymes and cells that produce PUFA-rich oils (due to its nutraceutical value), we used as a model three DGATs from the industrial oleaginous microalga Nannochloropsis oceanica (NoDGATs) that exhibited distinct FA-substrate preference: NoDGAT2A shows the highest preference for SFAs while 2C for PUFAs, resulting in high- and low-DU TAGs (and thus host cells), respectively, when overexpressed (14). Both NoDGAT2A and 2C were expressed in the yeast H1246 respectively and induced for TAG synthesis [eicosapentaenoic acid (EPA) was supplemented to 2C cells as substrates for producing PUFA-containing TAG; fig. S4, D and E, and Materials and Methods]. GC-MS of bulk cell mass revealed a higher DU of 2C cells (DU = 93.94; 36.73% SFA, 39.17% MUFA, and 24.09% PUFA) than 2A cells (DU = 45.14; 59.55% SFA, 34.55% MUFA, and 5.90% PUFA; Fig. 4A).

SCRS revealed that the ratio of 1655 cm⁻¹ (C=CH stretch) and 1445 cm⁻¹ (CH₂ bend) was distinct between TAG-synthetic activity of 2A cells (DU = 45.14; 59.55% SFA, 34.55% MUFA, and 5.90% PUFA; Fig. 4B):
**Fig. 3.** The pDEP-RADS of TAG-synthetic yeast cells. (A) GC-MS–based quantification of TAG levels extracted from bulk amounts of control cells (transformed with empty vector) or ScDGA1 cells. The total amount of TAG was normalized on the basis of that of total lipids. (B) Averaged SCRS of 60 control and 60 ScDGA1 cells. (C) Distribution of \( I_{1445} - I_{1800} \) of control and ScDGA1 cells separately. \( n > 100 \) in each of the three groups. (D) Sorting efficiency of pDEP-RADS, by comparison of relative abundance of target cells between “Sorted” and Waste pools. (E) Evaluation of viability of post-sorting cells. CFU, colony-forming units. (F) Performance of pDEP-RADS under various dilution factors in three replicate experiments. Unless specified, error bars indicate the SD of three independent experiments. **\( P < 0.01 \); n.s., no significant difference.

\((I_{1665} - I_{1800}) / (I_{11445} - I_{1800})\) was <1 for 2A while >1 for 2C cells, consistent with a higher DU of 2C cells. DU is linearly dependent on \( R^2 = 0.99\) on \((I_{1665} - I_{1800}) / (I_{11445} - I_{1800})\), underscoring accuracy of SCRS-based DU assessment (Fig. 4C). Consistently, \((I_{1665} - I_{1800}) / (I_{11445} - I_{1800})\) > 1.1 held for only ~5% of 2A cells yet ~68% of 2C cells (Fig. 4D), suggesting high specificity for sorting 2C cells. Moreover, the presence of a cell at the laser point was correlated with \( I_{2950} - I_{1800} > 600\), which thus serves to distinguish a cell from the background noise. Therefore, \((I_{1665} - I_{1800}) / (I_{11445} - I_{1800}) > 1.1 \) and \( I_{2950} - I_{1800} > 600\) was used to sort high-DU cells.

Using this DU-sorting criterion, post-induction NoDGAT2A and 2C cells were mixed and underwent pDEP-RADS. On average, purity of 2C cells was elevated from 16 to ~82% (Fig. 4E), of fivefold enrichment. Among the 20 randomly picked post-pDEP-RADS clones, 18 expressed the target gene of 2C, suggesting 90% sorting accuracy (Fig. 4F). Of the 80 randomly selected sorted cells (PUFA content quantified via GC-MS of bulk cell mass), 64 were confirmed carrying the target phenotype (80%; fig. S4F). Thus, pDEP-RADS can sort enzymes and cells for high DU.

**Culture-free, higher-throughput screening of previously unknown DGAT activity in vivo via pDEP-RADS**

Despite their vast volume in sequenced genomes and intensive interest (18), very few of DGAT gene candidates have been characterized for ex vivo or in vivo function, because of the highly tedious, low-throughput strategy for assaying the level and composition of TAG in the cell, where one gene at a time was screened including cloning, culture, polymerase chain reaction (PCR) identification, oil extraction, gas/liquid chromatography–mass spectrometry (GC/LC-MS)–based TAG composition profiling, etc. (Fig. 5A) (14, 19). For this reason, among the 11 candidate DAGT2s predicted in the *N. oceanica* genome, the in vivo TAG-synthetic activity has been validated for only 5 (2A, 2C, 2D, 2K, and 2J) (14, 19). We hypothesize that pDEP-RADS, since it is culture free, in parallel and
noninvasive, can greatly simplify and accelerate this process (Fig. 5B).
Specifically, after induction for TAG production, the library of cells after one-time transformation of the gene pool was sorted directly by pDEP-RADS; then, the sorted cells were identified for the encoded genes via sequencing.

For screening 10 genes, in one screening cycle, pDEP-RADS would only take ~5.5 days (Fig. 5C), ~300 USD in consumable cost (Fig. 5D) and ~1.5 human hours in labor cost (Fig. 5E). In contrast, the conventional approach would take ~31 days, ~1000 USD in consumable cost and ~25 human days in labor cost. As all genes were pooled together and transformed into cells at once, pDEP-RADS is insensitive to the increase in library size (Fig. 5C), ~1.5 human hours in labor cost (Fig. 5E). In contrast, the traditional methods such as TLC (14) or Nile Red staining have been unable to detect any difference between 2F/2H lines and the negative control (Fig. S4, G to J). Thus, pDEP-RADS is uniquely able to detect genes exhibiting weak TAG-synthetic activity, e.g., NoDGAT2F and 2H; whereas they were slightly higher than the empty vector control (Fig. 6D). Moreover, the percentage of TAG-producing cells (I_{2867} - I_{2879} > 0) was higher in 2F and 2H cells than the empty vector control (11.78 and 8.62 versus 5.37, P < 0.05), although lower than 2D cells (11.78 and 8.62 versus 24.50, P < 0.05; Fig. 6E). This strongly suggests in vivo TAG-assembly activity of the 2F and 2H enzymes.

Validation via GC-MS of the bulk mass of 2F- and 2H-expressing yeasts suggested that the TAG content of 2F and 2H cells was up to 103 and 59% from the empty vector control, although to a much lesser degree than 2A, 2C, and 2D cells (12,318, 645, and 4972% up from the control, respectively; Fig. 6F). In contrast, at this level of TAG content change, traditional methods such as TLC (14) or Nile Red staining have been unable to detect any difference between 2F/2H lines and the negative control (Fig. S4, G to J). Thus, pDEP-RADS is uniquely able to detect genes exhibiting weak TAG-synthetic activity, e.g., NoDGAT2F and 2H. Furthermore, for the sorted 2A, 2C, 2D, 2F, and 2H cells, the TAG content measured via SCRS and GC-MS is highly correlated (R^2 = 0.90; Fig. 6G), indicating that pDEP-RADS can accurately identify and sort TAG-synthetic yeasts and across a wide dynamic range, e.g., from 0.34% (2H) to 27.99% (2A) per total lipid.

Last, the DU of 2F and 2H cells was assessed via the ratios of 1655 and 1445 cm⁻¹ (Fig. 6H). "(I_{1665} - I_{1800}) / (I_{1445} - I_{1800})" of both
Fig. 5. The pDEP-RADS versus the culture-based methods in screening in vivo function of DGAT genes. (A) Traditional strategy to screening the in vivo activities of TAG-synthetic genes. pYES2.0 and NoDGAT genes (2A-2I) from N. oceanica were transformed separately into the yeast strain H1246, followed by plating, clone picking, PCR, and sequencing to identify the successfully transformed clones. The clones were then separately cultured, and then each underwent a period of induction to synthesize TAG. In the end, the TAG profile of each clone was analyzed by the tedious and time-consuming TLC and GC/LC-MS from the cultured biomass. (B) pDEP-RADS strategy. pYES2.0 and NoDGAT genes (2A-2I) were transformed simultaneously into strain H1246, and then induction and functional sorting ensued directly from the yeast library (without any plating). The two strategies were compared for time (C), consumable cost (D) and labor (E). In (C) and (D), the steps are (i) transformation, (ii) plating, (iii) liquid cultivation, (iv) PCR and Sanger sequencing, (v) induction, (vi) lipid extraction, (vii) TLC-based TAG isolation, and (viii) GC-MS analysis for the culture-based strategy, whereas (1) transformation, (2) induction, (3) pDEP-RADS, and (4) high-throughput sequencing (HT-seq) for the pDEP-RADS strategy. In (E), the steps are (i) transformation, (ii) plasmid extraction, PCR and electrophoresis-based target gene recovery, (iii) lipid extraction, (iv) TLC-based TAG isolation, and (v) GC-MS analysis for the culture-based strategy, whereas (1) transformation and (2) the pDEP-RADS procedure in the pDEP-RADS strategy.
2F and 2H cells were higher than 2A cells (1.05 and 1.15 versus 0.89; \( P < 0.05 \)), yet 2F cells lower than 2D cells (1.05 versus 1.14; \( P < 0.05 \)), with no difference between 2H and 2D observed (1.15 versus 1.14; \( P = 0.92 \); Fig. 6I). This indicated that both 2F and 2H are synthesizing TAG with high DU (while this activity of 2F was lower than 2D). GC-MS–derived DU values, at 2A < 2F < 2D and at 2A < 2H = 2D (Fig. 6I), validated this finding. The DU of all the sorted 2A, 2C, 2D, 2F, and 2H cells showed linear correlation between SCRS and GC-MS \(( R^2 = 0.99; \text{Fig. 6K}) \); thus, pDEP-RADS can accurately sort DU over a broad dynamic range. Together, these results established pDEP-RADS as a novel platform to screen enzymes and phenotypes that are not dependent on resonance Raman peaks (in this case, TAG content and DU), which greatly simplify and accelerate the process.

**DISCUSSION**

Screening of enzyme libraries for in vivo activities is frequently a rate-limiting step in the design of enzymes as well as microbial cell factories \((20, 21)\). Being single-cell-level, label-free, noninvasive,
and richly informative, RACS technologies address the limitations of GC/LC–MS–based methods (14, 19) and FACS (22–26), thus are finding expanding applications in microbiome research and synthetic biology (1). However, existing RACS systems are either of low throughput (4–9) or not applicable for sorting phenotypes associated with nonresonance Raman peaks (which represent the majority of peaks in an SCRS) (10–12). Here, by (i) exerting a periodical pDEP force to trap and arrange fast-moving single cells via an angled [rather than flatted (11)] electrode array for sufficient time for efficient SCRS acquisition; (ii) applying SCRS-based microdroplet sorting to automatically sort target cells with high accuracy; (iii) selecting quartz with low Raman background as the structural material of the pDEP-RADS chip to further increase the efficiency of SCRS acquisition and accuracy of target cell sorting, we developed a fully automated pDEP-RADS system that solved this conflict.

Using pDEP-RADS, microalgal DAGTs were sorted in a faster, simpler, cheaper, and label-free manner for both TAG level and DU features with substantial throughput (~120 cells min−1 for TAG-synthetic cells and ~40 cells min−1 for EPA-accumulating cells) and high accuracy (>95% for TAG-synthetic cells and >80% for EPA-accumulating cells). This represents orders of magnitude improvement in time and cost efficiency. Two previously unknown enzyme variants that produce low but substantial amounts of MUFA-rich TAG were discovered by pDEP-RADS, but not by Nile Red staining or GC-MS of bulk cell mass. Sorting of these genes by FACS are limited by the nonspecific binding of fluorescent labels [e.g., Nile Red and Boron-dipyrromethene (BODIPY)], the poor cell wall permeability of many fluorescent probes, the difficulty in quantitation and the inability to sort based on DU (27).

To our knowledge, this is the first demonstration of RACS for enzyme discovery. The range of enzyme activity that can be tackled by pDEP-RADS is broad. For example, as many high-value compounds in the cell carry nonresonance (e.g., starch, protein, and nucleic acids) or resonance Raman signal (e.g., pigments) (1, 28), pDEP-RADS can be extended to mining or de novo design of the numerous synthetic enzymes or cells. As a generally applicable single-cell “phenome” (1), SCRS can also distinguish or model bacterial species (6), general or substrate-specific metabolic activity (5), intracellular levels of starch and protein (15), drug sensitivity (29, 30), cross-species metabolite exchange (31), etc.; most of these phenotypes are associated with nonresonance Raman peaks and thus can take advantage of pDEP-RADS. Therefore, by enabling nonresonance Raman peak-based sorting, pDEP-RADS greatly expands the application scope of RACS.

In addition, the droplet-sorting feature of pDEP-RADS brings several advantages. Although continuous flow–based sorters can accomplish cell sorting with ultrahigh throughput and accuracy (32, 33), the resulted cell stream disturbance may affect efficiency of Raman interrogation, especially for small cells (11, 32). In contrast, droplet sorting allows not just high-throughput screening of the target cells (22–25) but the maintenance of a stable cell stream (12). Moreover, as the throughput of droplet sorting far outpaces spontaneous Raman acquisition, pDEP-RADS systems of even higher throughput, e.g., by coupling droplet sorting to surface enhanced (34) or “coherent” Raman [e.g., coherent anti-Stokes and stimulated Raman scattering (35–37)], can be developed. Furthermore, different from continuous flow–based sorters (32, 33) or additional RACS family members such as Raman tweezers (4, 5, 9), RACE (6–8), or RAMS (10, 11), the pDEP-RADS sorted cells are already droplet compartmentalized, which facilitates seamless integration with downstream applications such as extraction of intracellular DNA, RNA, proteins, and metabolites for single-cell sequencing.

On the other hand, it is notable that the stable running time of pDEP-RADS at its current form is still relatively short (~30 min), which is mainly caused by the precipitation of cells under low flow rates. This can pose a problem for the screening of large mutant libraries. One potential solution is to enlarge the width of channel to allow a high loading flow rate to avoid the in-tube precipitation yet maintain a low in-channel flow rate for efficient single-cell trapping. In addition, a magnet disc can be used to avoid precipitation in syringe or bottle (9). Furthermore, to maintain high single-cell trapping and alignment efficiency in a wide channel, an external force [e.g., acoustic standing waves (35, 37)] can be applied to focus the cells into a stream that flows through the electrodes and laser point. With these designs, a pDEP-RADS device with long-term operating stability can be developed. As a result, novel RACS instruments based on pDEP-RADS should emerge soon for efficiently establishing single-cell phenotype-genotype links or high-throughput mining of enzymes (or other functional molecules), in a culture-free and label-free manner.

MATERIALS AND METHODS

Rational design of yeast cells

The NoDGAT-expressing yeast strains were generated as reported (14). Briefly, S. cerevisiae strain H1246 with knockouts of DGA1, LRO1, ARE1, and ARE2 (17) was used as the host cells for in vivo enzyme activity screening and maintained on yeast extract peptone dextrose plates solidified with 2% agar. The yeast DGA1 gene and N. oceanica genes of NoDGAT2A and 2C were subcloned into pYES2.0 vector (Invitrogen) and transformed into H1246, using the lithium acetate procedure (38), to construct the TAG (+) (ScDGA1), SFA-accumulating (NoDGAT2A) and PUFA (EPA)–accumulating (NoDGAT2C) cells. In addition, the empty vector pYES2.0 was transformed into H1246 to construct TAG (−) cells, as the negative control. For screening a library of enzymes, the empty vector pYES2.0 and the NoDGAT2A-2I genes were transformed simultaneously (i.e., as a premixture of vector DNA) into H1246. For culture-based screening, transformants were then selected by plate culture of selective (SC) minimal medium (0.67% yeast nitrogen base and 2% glucose; solidified with 2% agar) containing 0.074% -His/-Ura DO supplement (Supplementary Materials and Methods).

Cell culture and induction for TAG production

Yeast cells were cultured and prepared as reported (14). Briefly, the constructed strains, including TAG (+) (ScDGA1) cells, TAG (−) (pYES2) cells, SFA-accumulating (2A) cells, and PUFA (EPA)–accumulating (2C) cells, were maintained at 30°C and stored at 4°C on SC minimal medium solidified with 2% agar. To induce cells to produce TAG, ScDGA1 and pYES2 cells (harvested by centrifugation after culture in SC medium) were cultured in induction medium (0.67% yeast nitrogen base, 0.074% -His/-Ura DO supplement, 1% raffinose, and 2% galactose) for 3 days. To induce the cells to accumulate SFA-rich TAG, harvested 2A cells were similarly cultured. To induce the cells to accumulate PUFA (EPA)–rich TAG, harvested 2C cells were cultured in modified induction medium [supplemented with 90 μM EPA in the presence of bovine serum albumin (0.1 g/liter)] for 3 days. Before pDEP-RADS, the induced
cells were harvested and washed with sterile deionized water under 3000g. Cell concentration was measured using a cell count plate and was adjusted to ~7.63 \times 10^5 and ~2.5 \times 10^6 cells ml⁻¹ for the screening of TAG-producing and PUFA (EPA)-accumulating cells separately. Then, the target cells were mixed with nontarget cells at a specific ratio for pDEP-RADS, which was later determined by sampling >100 cells via the Raman Points Mapping mode.

**Lipid isolation and quantification from bulk cells to validate the single-cell sorting results**

Total lipids were extracted from dried samples using chloroform/methanol (2:1 v/v) with 100 mM internal control tri13:0 TAG (Sigma-Aldrich, USA) and separated on a silica TLC plate using a mixture of solvents consisting of petroleum ether, ethyl ether, and acetic acid (70:30:1, by volume). To quantify the amount of TAG accumulated in yeasts expressing the NoDGAT2 constructs, we scraped TAG bands from the TLC plate. FA methyl esters (FAMEs) were prepared by acid-catalyzed transmethylation of the TAG bands and then analyzed by GC-MS as previously described (39). Mixed analytical standards of FAMEs and pentadecane were used as external and internal standard, respectively. The amounts of TAGs and the profiles of TAG-associated FA were calculated on the basis of GC-MS results.

**Design and fabrication of the pDEP-RADS chip**

The pDEP-RADS chip uses quartz as the structural material. Both the bottom ITO electrode array layer and the top channel layer (Fig. 1B) were designed using AutoCAD 2013 (Autodesk, USA). The six electrodes were designed as 80 nm in height, 25 μm in width, and 25 μm in gap. The channels for loading cells and Raman acquisition were designed as 45 μm in width and 10 μm in height. The pDEP-RADS chip was fabricated via standard manufacturing processes by wet etching. After bonding, the device was heated to 100°C on the hot plate, and a low-melting point In-Sn solder was filled into the sorting electrode channels. Small pieces of copper wire were inserted to make the electrical connection with the solder electrodes. Last, the chip was treated with 0.5% trichloro (1H, 1H, 2H, 2H-perfluoro-ocetyl) silane in ethanol for 15 min to generate a hydrophobic surface for droplet generation (additional details of chip fabrication in fig. S5A and Supplementary Materials and Methods).

**System setup of pDEP-RADS**

As shown in Fig. 1C, to avoid their sedimentation during loading, cells were stored in the PEEK tubing with a small inner diameter (304.8 μm; Cole-Parmer, USA), which was further vertically connected to the microfluidic device and the syringe equipped on the pumps (LSP01-2A, Longer Pumps, China). Because of its extraordinary biocompatibility and high oxygen permeability and stability, the fluorinated oil of Pico-Surf 1 (2% in Novec 7500, Dolomite, UK) was used for droplet generation.

Ac voltages of 16 Vp-p at frequencies of 10 MHz for dielectrophoretic cell trapping were generated by an arbitrary function generator (DG4620, RIGOL Ltd., China). The outputs of the function generator were connected to two pads of the electrodes on the microfluidic chip via conductive tapes. A sinusoid wave (output CH1) was modulated by a pulsed wave (output CH2) to generate a periodic ac field (fig. S5, B and C) to automatically trap and release cells. A normally on relay was connected between the function generator and the electrodes of the microfluidic chip and was activated by a digital input/output (I/O) unit (DIO-1616LX-USB, CONTEC Ltd., USA) to trigger immediate interruptions on pDEP for the on-demand release of cells. A high-voltage amplifier controlled by the same digital I/O unit was used to generate DEP to trigger the target droplet sorting.

Raman microscopy was carried out on RACS-Seq (Qingdao Single-cell Biotechnology Co., Ltd., China) or a customized LabRAM HR (Horiba, France). The RACS-Seq instrument is equipped with a Nd:YAG (yttrium-aluminum-garnet) 532-nm laser emitter as the excitation light source (the laser power of 300 mW was used in all the experiments), an EMCCD (Newton DU970N-BV, Andor, UK) for collecting SCRS, a high-speed CCD camera (Pike F-032, Allied Vision Technologies, China) for monitoring the cell and droplet flow, and a 60× water objective (numerical aperture = 1.0, Olympus, UK) to focus the laser beam on the sample. A 300 lines mm⁻¹ grating was used for the measurements, resulting in a spectral resolution of ~2 cm⁻¹. A 660-nm light-emitting diode array was used as the light source for monitoring the sorting process.

A software QSpec was developed to control the electronics (EMCCD, function generator, relay and high-voltage amplifier, etc.) and adjust the system parameters (e.g., acquisition time, trap and release frequency, and DEP duration). With QSpec, all the RADS units including microfluidic device, Raman system, function generator, and high-voltage amplifier were integrated and can be operated in an automatic mode.

**Evaluating the system stability of SCRS acquisition**

PS beads with an identical size of 10 μm in diameter were used to evaluate the stability of SCRS acquisition. Although the beads are not sensitive to the pDEP force, the strong intensity of their Raman peaks allows the acquisition of the Raman spectra under the trap-free condition in the flow mode. Specifically, the PS beads were loaded into the pDEP-RADS chip without a pDEP-based trap. The acquisition time was set as 1 ms for the raw Raman spectra of PS beads. The intensity at 1001.1 cm⁻¹ (one of the most prominent peaks of PS beads; defined as \(I_{1001} - I_{1800}\)) were extracted from the raw Raman spectra of PS beads (\(n > 100\)) and its SD was derived to evaluate the stability of Raman acquisition.

**Recovery, validation, and evaluation of the post-pDEP-RADS cells**

To validate sorting accuracy, the sorted droplets containing target cells were collected and dropped on the surface of CaF₂, so that the fluorinated oil would evaporate quickly at room temperature. Then, a drop of water was added, followed by covering a quartz slice. The SCRS of the sorted cells were acquired and analyzed to verify whether the sorted cells indeed met the predesignated sorting criteria. For the PCR assays, the sorted droplet containing target cells were directly poured on the SC plate and incubated at 30°C. Then, 20 clones for each assay were randomly selected and cultured in liquid SC medium at 30°C and 180 rpm, followed by gene extraction, PCR, and electrophoresis. To evaluate the vitality of post-sorting cells, the sorted droplets that contain target cells were directly poured on the SC plate and cultured at 30°C for 24 hours; then, the clones were counted manually. Since this assay aims to assess the vitality of post-sorting cells, the sorting criterion was set as \(I_{1260} - I_{1800} > 150\) (the chip background is <60), under which all the cells would be sorted. The unsorted cells were used as the control group.

To validate the TAG-synthetic activity of post-pDEP-RADS cells from the yeast libraries (derived by one-time transformation of the candidate DGAT gene pool), one aliquot of the sorted cells was...
plated (Fig. 5B) and >200 colonies were randomly picked and then identified by Sanger sequencing (Fig. 6C). Then, three colonies from each of the sorted 2A, 2C, 2D, 2F, and 2H cells were cultured and induced separately (i.e., for TAG production). For each such colony-derived culture (in three replicates), two kinds of analyses were performed in parallel: (i) >1000 individual cells were analyzed via SCRS on a calcium fluoride slide (Fig. 6D) to derive the percentage of TAG-synthetic cells via \( I_{3286}^* / I_{2879} > 0 \) (Fig. 6E), averaged TAG content of the colony via \( I_{2867} / I_{2879} \) (Fig. 6G), and averaged DU of the colony via \( \left( I_{1445} / I_{1800} \right) / \left( I_{1445} / I_{1800} \right) \) (Fig. 6, H, I, and K); and (ii) the yeast-cell populations were analyzed via GC-MS for TAG content (Fig. 6F) and DU at the bulk cell level (Fig. 6, J and K). Last, the correlation of TAG content and DU between the results derived from SRS and GC-MS was probed (Fig. 6, G and K).

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/32/eabb3521/DC1

**REFERENCES AND NOTES**

1. Y. He, X. Wang, B. Ma, J. Xu, Ramanome technology platform for label-free screening of microalgal cells. *Biotechnol. Adv.* 37, 107388 (2019).

2. Y. Song, H. Yin, W. E. Huang, Raman activated cell sorting. *Curr. Opin. Chem. Biol.* 33, 1–8 (2016).

3. Q. Zhang, P. Zhang, H. Gou, C. Mou, W. E. Huang, M. Yang, J. Xu, B. Ma, Towards high-throughput multifluoride Raman-activated cell sorting. *Analyst* 140, 6163–6174 (2015).

4. W. E. Huang, A. D. Ward, A. S. Whiteley, Raman tweezer sorting of single microalgal cells. *Environ. Microbiol.* Rep. 1, 44–49 (2009).

5. D. B. Eddy, M. D. Lee, D. Woebcken, Y. Wang, D. Zhu, M. Palatsinsky, A. Schintlmeter, M. C. Schmid, B. T. Hanson, N. Shetzer, I. Mizrahi, I. Rauch, T. Decker, T. Bocklitz, J. Popp, C. M. Gibson, P. W. Fowler, W. E. Huang, M. Wagner, Tracking heavy water (D,O) incorporation for identifying and sorting active microalgal cells. *Proc. Natl. Acad. Sci. U.S.A.* 112, E194–E203 (2015).

6. Y. Wang, Y. Ji, E. S. Wharfe, R. S. Meadows, P. March, R. Goodacre, J. Xu, W. E. Huang, Raman activated cell ejection for isolation of single cells. *Anal. Chem.* 85, 10697–10701 (2013).

7. Y. Song, A.-K. Kaster, J. Vollmers, Y. Song, P. A. Davison, M. Fenretrou, G. M. Preston, I. P. Thompson, J. C. Murrell, H. Yin, C. N. Hunter, W. E. Huang, Single-cell genomics based on Raman sorting reveals novel carotenoid-containing bacteria in the Red Sea. *J. Microbiol. Biotechnol.* 10, 125–137 (2017).

8. X. Jing, H. Gou, Y. Gong, X. Su, L. Xu, Y. Ji, Y. Song, I. P. Thompson, J. X. Yu, W. E. Huang, Raman-activated cell sorting and metagenomic sequencing revealing carbon-fixing bacteria in the ocean. *Environ. Microbiol.* 20, 2241–2255 (2018).

9. K. S. Lee, M. Palatsinsky, F. C. Pereira, J. Nguyen, I. F. Fernandez, A. J. Mueller, F. Menolascina, H. Daims, D. B. Eddy, M. Wagner, R. Stocker, An automated Raman-based platform for the sorting of live cells by functional properties. *Nat. Microbiol.* 4, 1035–1048 (2019).

10. D. McIlvenna, W. E. Huang, P. Davison, A. Glidle, J. Cooper, H. Yin, Continuous cell sorting in a flow based on single cell resonance Raman spectra. *Lab Chip* 16, 1420–1429 (2016).

11. P. Zhang, L. Ren, X. Zhang, Y. Shan, Y. Wang, Y. Ji, H. Yin, W. E. Huang, J. Xu, B. Ma, Raman-activated cell sorting based on dielectricophoretic single-cell trap and release. *Anal. Chem.* 87, 2282–2289 (2015).

12. X. Wang, L. Ren, Y. Su, Y. Ji, Y. Liu, C. Li, X. Li, Y. Zhang, W. Wang, Q. Hu, D. Han, J. Xu, B. Ma, Raman-activated droplet sorting (RADS) for label-free high-throughput screening of microalgal single-cells. *Anal. Chem.* 89, 12569–12577 (2017).

13. M.-H. Liang, J.-G. Jiang, Advancing oleaginous microorganisms to produce lipid via metabolic engineering technology. *Prog. Lipid Res.* 52, 395–408 (2013).

14. Y. Xin, Y. Lu, Y.-Y. Lee, L. Jia, J. Q. Wang, D. Wang, F. Bai, H. Hu, Q. Hu, J. Liu, Y. Li, J. Xu, Designing producer oils in industrial microalgae by rational modulation of co-evolving type 2 diacylglycerol acyltransferases. *Mol. Plant* 10, 1523–1539 (2017).

15. Y. He, P. Zhang, S. Huang, T. Wang, Y. Ji, J. Xu, Label-free, simultaneous quantification of starch, protein and triacylglycerol in single microalgae cells. *Biotechnol. Biofuels* 10, 275 (2017).
39. Q. Zhang, H. K. Chieu, C. P. Low, S. Zhang, C. K. Heng, H. Yang, Schizosaccharomyces pombe cells deficient in triacylglycerols synthesis undergo apoptosis upon entry into the stationary phase. J. Biol. Chem. 278, 47145–47155 (2003).

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Competing interests: J.X. and B.M. are on the scientific board of Qingdao Single-cell Biotechnology Co., Ltd. The authors declare that they have no other competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested from the authors.

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