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Towards high-throughput microfluidic Raman-activated cell sorting

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Raman-activated cell sorting (RACS) is a promising single-cell analysis technology that is able to identify and isolate individual cells of targeted type, state or environment from an isogenic population or complex consortium of cells, in a label-free and non-invasive manner. However, compared with those widely used yet labeling-required or staining-dependent cell sorting technologies such as FACS and MACS, the weak Raman signal greatly limits the further development of the existing RACS systems to achieve higher throughput. Strategies that can tackle this bottleneck include, first, improvement of Raman-acquisition efficiency and quality based on advanced Raman spectrometers and enhanced Raman techniques; second, development of novel microfluidic devices for cell sorting followed by integration into a complete RACS system. Exploiting these strategies, prototypes for a new generation of RACS have been demonstrated, such as flow-based OT-RACS, DEP-RACS, and SERS/CARS flow cytometry. Such high-throughput microfluidic RACS can provide biologists with a powerful single-cell analysis tool to explore the scientific questions or applications that have been beyond the reach of FACS and MACS.

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1. Introduction

Single-cell analysis is a frontier of life science and biotechnology. Understanding biological heterogeneity at single-cell resolution can shed light on the molecular mechanisms that underlie cellular differentiation and development, origin and microevolution of cancer, and contribution of environmental microbiota.^{1–3} The most desired single-cell analysis tools are expected to sample and interrogate cells in parallel in their native state, *e.g.*, in a non-invasive, label-free, and high-throughput manner. However, single-cell analysis tools that are widely used at present, such as fluorescence-activated cell sorting (FACS) and magnetic-activated cell sorting (MACS), typically require labeling of the pre-sorted cells with fluorescent or magnetic molecules and compounds, which may affect or even alter the “natural” state of the cells despite their very high throughput. Therefore, the paucity of high-throughput technologies for label-free and non-invasive functional

sorting of cells has been one key bottleneck that limits the scientific potential of single-cell analysis and hinders its wider biotechnological application.^{4–6}

Raman spectroscopy, especially confocal Raman microspectroscopy, has been employed for analyzing biological samples in a label-free and non-invasive fashion.^{7–9} Moreover, during the last decade, single-cell Raman spectroscopy (SCRS) has emerged which is able to provide a wide spectrum of biochemical information of individual living cells in a quantitative manner, thus making it possible to identify the types, physiological states, and environmental conditions of the cell.^{10–15} Furthermore, as illustrated in Fig. 1, SCRS-based cell phenotyping can be coupled with a number of single-cell manipulation technologies such as optical tweezers (OT)^{16,17} and laser ejection¹⁸ to subsequently accomplish isolation of the target cell, *i.e.*, Raman-activated cell sorting (RACS).^{16–22} RACS is a promising technology with key advantages, being free of labeling, non-invasiveness and capability of simultaneous analysis of multiple features or phenotypes, as comparable with either FACS or MACS. However, one weakness of the existing RACS systems has been the quite low throughput, mainly due to the intrinsically weak Raman signal of a single cell and the manual mode of operation. Recently a number of advancements, including microfluidics-based cell manipulation techniques (*e.g.*, flow-based OT-RACS,^{17,20} and DEP-RACS²¹) and advanced Raman techniques (*e.g.*, SERS flow cytometry,^{23–25} RRS cell counting,²⁶ and CARS flow cytometry^{27,28}), have been integrated into RACS systems, which

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Fig. 1 Schematics from SCRS (single cell Raman spectroscopy) to RACS (Raman-activated cell sorting) then towards HT-RACS (high-throughput RACS).

have improved the throughput of RACS and with further development might usher in high-throughput RACS (HT-RACS).

In this minireview, the state-of-the-art and challenges of RACS are summarized. Potential approaches to increase the throughput of RACS are reviewed, including the various technologies to enhance the quality and rate of SCRS acquisition and to improve the microfluidic cell-processing coupled to SCRS measurement. It is prospected that, with these advances, high-throughput microfluidic RACS will emerge in the not-so-distant future.

2. Single-cell Raman spectroscopy (SCRS)

Single-cell Raman spectroscopy (SCRS) can produce an intrinsic “fingerprint” of biochemical components in an individual cell, such as nucleic acids, proteins, carbohydrates, and lipids.^{10–15} A number of studies have demonstrated the ability of SCRS to distinguish cells based on cell types, physiological states, and environmental conditions (Fig. 2A). Chan *et al.*²⁹ firstly demonstrated that the feasibility of confocal laser tweezers Raman spectroscopy to rapidly identify single bacterial spores from a mixture of particles containing polystyrene and silica beads in aqueous solution (Fig. 2B). Subsequently, SCRS has been developed to discriminate different bacteria^{30–32} and discern cancerous cells^{33–35} in combination with statistical analysis. Changes in physiological states of cell stages^{36–45} or activity patterns⁴⁶ usually involve fluctuations in both concentrations and distributions of cellular components, thus can also be detected by SCRS. Shigeto and coworkers^{37,38} revealed dynamic changes of cellular phospholipids, proteins and polysaccharides during the cell cycle of a yeast cell by *in vivo* multi-mode Raman imaging (Fig. 2C). Other studies have shown that differentiation statuses of human stem cells^{39–41} and different stages of cancer cells^{42–44} can be assessed by means of Raman micro-spectroscopy at the single cell level. Furthermore, alteration in environmental conditions such as the availability and level of nutrients,^{47–49} antibiotics/drugs,^{50–54} or stress from chemicals (*e.g.*, glyoxal,⁵⁵ butanol,⁵⁶ hydroxyl radical,⁵⁷ nanoparticle⁵⁸) or physical conditions (*e.g.*, heat,⁵⁹ light,⁶⁰ temperature,^{61,62} hyperosmotic stress⁶³) can induce variations in

biochemical composition of cells, leading to distinguishable SCRS signals (Fig. 2D). For example, oleaginous microalgal cells under nitrogen-depletion conditions can be discriminated from those under the nitrogen-repletion conditions with over 93% accuracy, as early as six hours after the change in culture conditions.⁴⁸ These are just a few examples that highlight the potential application of RACS in discriminating and then isolating individual cells of a particular type, state or environment.

3. From SCRS to RACS

Raman-activated cell sorting (RACS) is being developed as a promising technology for functional sorting of cells.^{16–22} RACS is complementary to FACS or MACS, due to the label-free and non-invasive nature of SCRS. In addition to the acquisition of SCRS signal, a cell manipulation technique is employed in an RACS system to perform the separation of target cells from the cell population. In the first-generation RACS platforms, two laser-assisted techniques have been demonstrated with good integrating ability and compatibility with SCRS acquisition. One of them is optical tweezers, a widely-used cell/particle manipulation technique based on the radiation pressure force of a focused optical beam, which can be combined with Raman spectroscopy to form a laser tweezers Raman spectroscopy (LTRS) system.^{64,65} In the LTRS-based RACS platform, the optical tweezers firstly trapped a single cell from the bulk cell-suspension and acquired SCRS signals for the purpose of functional classification or identification, then the cell of interest was moved and separated from the cell suspension for physical isolation and collection (Fig. 3A).^{16,66} It has been demonstrated that this platform can non-invasively isolate yeast and bacterial cells for further cultivation and single-cell genome amplification.⁶⁶ The other technique is Raman-activated cell ejection (RACE) that exploited the mechanism of laser-induced forward transfer (LIFT).¹⁸ In a RACE system, single cells of interest as identified by SCRS on a slide (coated with a light-adsorbing interlayer) can be readily ejected from the solid substrate and collected into a prepared microcontainer *via* a 337 nm pulsed laser (Fig. 3B).¹⁸

Although the concept of RACS has been proved by the first generation of RACS platforms, these prototypes operate in a

Fig. 2 Single-cell Raman spectroscopy (SCRS). (A) SCRS for probing cell types (B), physiological states (C), and environmental conditions (D). (B) Identification of a single *Bacillus cereus* spore from a mixed sample by SCRS. Adapted with permission from ref. 29. Copyright (2004) American Chemical Society. (C) Raman imaging of a single yeast cell during the cell cycle. Adapted with permission from ref. 38. Copyright (2012) American Chemical Society. (D) Red-shift appearing in certain Raman spectra of *P. fluorescens* SBW25 when substituting cellular biomass ^{12}C with ^{13}C . Adapted with permission from ref. 30. Copyright (2004) American Chemical Society.

Fig. 3 The first-generation RACS platforms. (A) Optical tweezer based RACS. Adapted with permission from ref. 16. Copyright (2005) Optical Society of America. (B) Raman-activated Cell Ejection (RACE). Adapted with permission from ref. 18. Copyright (2013) American Chemical Society.

semi-continuous mode due to the long time required for acquiring the intrinsically weak Raman signal and the manual operation, in which cells are intermittently identified and

manually sorted one by one. Consequently, the existing RACS setups have suffered from a drawback of low throughput. However, high-speed single-cell analysis and sorting is always

desired since a real biological sample usually contains a huge amount of cells (e.g., several million cells in 1 mL human blood, and for comparison purpose, the throughput of the present-day commercial FACS can reach >100 000 analysis events per second and >70 000 sorting events per second). Thus development of high-throughput RACS (HT-RACS) is one crucial front of single cell analysis.

4. Towards high-throughput RACS

Two challenges should be taken into account in order to accomplish high-throughput RACS: one is to improve quality and rate of SCRS acquisition, and the other is to expedite cell isolation through changing the slow semi-continuous sorting mode to a fast continuous mode. A number of techniques can potentially help to tackle these two challenges. Regarding SCRS acquisition, on the one hand, higher sensitivity of Raman detection could be achieved by employing advanced Raman techniques. On the other hand, microfluidics-based cell manipulation techniques (e.g., cell trap-release) can also improve the quality of SCRS and thus elevate the efficiency of SCRS acquisition. As for the cell isolation procedure, a fast continuous mode could be produced in a microfluidic device designed for isolation of target cells from a cell flow. Moreover, microfluidics can serve as an open platform for integrating all these techniques for high-throughput RACS. Here, the state-of-the-art of these techniques is first summarized and proof-of-concept experiments that integrated these technologies for high-throughput RACS are then presented.

4.1. SCRS acquisition for high-throughput RACS

4.1.1. Advanced Raman techniques to improve the sensitivity of Raman detection

Advanced Raman spectrometer. Over the past few decades, significant advancements have been made in Raman instru-

mentation, involving the various aspects of laser sources, optical filters, photon detectors, *etc.*, to increase the sensitivity of Raman detection and to facilitate the expanding bio-analytical applications.^{67–69} For example, a Raman spectrometer system was optimized to improve Raman spectral quality, by shortening the Raman light path, employing a low noise and sensitive EMCCD (Electron-Multiplying Charge-coupled Device) for Raman signal detection, and increasing the incident laser power.¹⁸ As a result the acquisition time of satisfactory SCRS for bacterial cells was reduced to the millisecond level (~100 ms).

Enhanced Raman techniques. At the same time, a variety of novel enhanced Raman techniques have been developed to improve the sensitivity of Raman detection. Surface-enhanced Raman spectroscopy (SERS, Fig. 4A) is an enhanced Raman technique in which Raman signals of molecules in the vicinity of rough metal (typically Ag and Au) surfaces or nanoparticles can be amplified by as much as 10–14 orders of magnitude.⁷⁰ Two kinds of SERS techniques have already been exploited for cell imaging and detection with ultrahigh sensitivity. One is to enhance the Raman signals of chemical components on cell surface or inside the cell, as probed by “naked” nanoparticles (without Raman reporter molecules), for cell detection and identification.^{71–76} For instance, Kneipp *et al.* demonstrated the feasibility of using colloidal gold to ultra-sensitively probe cellular endosomal compartments,⁷² and deliver strong SERS signals of intrinsic biomolecules inside a living cell.⁷¹ The other is to provide highly-sensitive Raman signals for indirect cell detection using SERS tags that consist of nanoparticles and Raman reporter molecules.^{77,78} It has been shown that cancer cell detection and recognition can be accomplished by using SERS tags to target specific biomarkers expressed on the surface membrane of cancer cells.^{25,79–82} Other studies have presented SERS tags as high-sensitive pH sensors to report the pH values in live cells *via* delivering SERS signals of specific Raman reporters.^{83–87}

Fig. 4 Enhanced Raman techniques. (A) Surface-enhanced Raman spectroscopy (SERS). Adapted from ref. 82 (Copyright (2014), with permission from Elsevier). (B) Resonance Raman spectroscopy (RRS). Adapted from ref. 89 with permission of The Royal Society of Chemistry. (C) Stimulated Raman spectroscopy (SRS). Adapted from ref. 92 (Copyright (2011), with permission from Elsevier). (D) Tip-enhanced Raman spectroscopy (TERS) (adapted with permission from ref. 97. Copyright 2012 John Wiley and Sons).

Resonance Raman Spectroscopy (RRS, Fig. 4B) is a selective Raman enhancement technique, in which Raman scattering intensities of specific molecules can be enhanced by 4–6 orders of magnitude without the need for labels and sophisticated equipment, when an electronic transition of the molecule matches or is close to the frequency of the applied incident light.⁸⁸ At present, RRS is mainly used in the analysis of pigment-containing cells such as photosynthetic cells and red blood cells.^{26,89–91} For instance, the Raman acquisition time for a single photosynthetic microorganism can be reduced to 1 ms.²⁶ Coherent anti-Stokes Raman spectroscopy (CARS) and stimulated Raman spectroscopy (SRS, Fig. 4C) both originate from a nonlinear coherent Raman phenomenon when the frequency difference between a pump beam (ω_p) and a Stokes beam (ω_s) matches a Raman-active vibrational mode, and can achieve 3–5 orders of Raman signal enhancement.^{92,93} Unlike CARS with the nonresonant background and spectral distortion, SRS has been utilized for high-sensitivity, label-free, and quantitative imaging of live cells in several cases.^{94–96} One of the characteristics of CARS/SRS is that the signal enhancement always focuses on only one spectral line. Tip-enhanced Raman spectroscopy (TERS, Fig. 4D) can enhance the weak signal of spontaneous Raman spectroscopy and achieve high lateral resolution based on the intrinsic properties of plasmonic structures at the apex of a metallic or metal-coated tip.⁹⁷ It could be used to investigate complex biological samples such as virus particles, bacteria, human cells, *etc.*, with ultrahigh sensitivity for single-molecule detection and nanoscale spatial resolution.^{98–100}

“Raman labels” technique. Raman spectroscopy can provide rich information of biological specimens in a label-free manner. On the other hand, the utilization of Raman labels (*i.e.*, Raman tags) can certainly expand the range of applications of Raman in biomedical analysis. In the “Raman labels” technique, strong and sensitive signals of Raman tags (*e.g.*, alkyne, deuterium, metal–carbonyl complex) can be produced in the silent region of Raman spectra of cells, which have been used in Raman imaging of small target molecules inside cells.^{101–104} For example, Mikiko Sodeoka *et al.* presented the feasibility of an alkyne tag to probe the localization of EdU (5-ethynyl-2'-deoxyuridine, which can be incorporated into cellular DNA during cell proliferation) in cell nucleus and meanwhile monitor DNA synthesis of living HeLa cells by Raman spectroscopy.¹⁰¹ Recently, Michael Wagner *et al.*⁴⁶ revealed the incorporation of deuterium-tagged D₂O into active microbial cells by using Raman spectroscopy. When further combined with optical tweezers, active microbial cells labelled with heavy water could be identified and sorted. Therefore, Raman labels as spectral markers can be introduced in RACS to increase the specificity and sensitivity of target cell identification by Raman spectra.

4.1.2. Cell manipulation techniques to improve efficiency of SCRS acquisition. In a continuous flow, accurate alignment between the single cells and the Raman detection spot is crucial for high-efficiency SCRS acquisition. It remains a technical challenge to achieve the alignment solely by sheath flows

(such as that in FACS), due to the much smaller Raman detection spot (*e.g.*, with a typical diameter of 1–2 μm when interrogating bacterial cells). Microfluidics-based cell manipulation techniques (such as cell trap–release) could achieve precise cell alignment and thus improve the efficiency of SCRS acquisition. In the cell trap–release strategy, an individual cell can be trapped accurately on the Raman detection spot for signal acquisition before being released by periodically applying a trapping force. As one of the forces that can be exploited for such cell trap–release, optical tweezers offer a contactless trapping force to hold a small particle or living cell in a fluidic medium (Fig. 5A), with its advantage being minimal sample contamination (optical tweezers have been used in the above mentioned LTRS system for trapping an individual cell for acquiring its SCRS).^{34,64,65} Similarly, an optical waveguide, with the aid of its near-field optical forces, can be utilized for trapping single particles or several particles in parallel, for example micro-/nano-particles, cells, DNA molecules.^{105,106} Furthermore, it is feasible to combine optical waveguides with Raman spectroscopy on a microfluidic device for on-line particle per cell trapping and identification.^{107,108} Dielectrophoresis (DEP) can provide a trapping force (ranging from 0.1 nN to 1 nN) by applying a nonuniform electric field on cells (Fig. 5B).^{109,110} The precise cell alignment by DEP cell trap–release has just been demonstrated, where 75% of the trapped cells from a continuous cell flow could be coincident with the laser detection spot and then identified by SCRS.²¹ Yet another potential force that can trap–release cells is optoelectronic tweezers (OET), which combines the advantages of optical tweezers and DEP by a light induced dielectrophoresis mechanism, and thus can manipulate particles or cells with high resolution and high throughput simultaneously (Fig. 5C).^{111,112} An OET-based microfluidic platform has been developed for single cell sample preparation through trapping, isolating, and exporting cells individually.¹¹³

4.2. Microfluidics-based cell isolation for high-throughput RACS

A number of microfluidic cell sorting approaches have been reviewed recently.⁶ Herein we only introduce those approaches that are more likely to be exploited in high-throughput RACS (Table 1). (i) Electrophoresis for cell isolation relies on the migration of negatively charged cells to the positive electrode, caused by electrostatic effects, which has been used for sorting cells and water-in-oil droplets under continuous flow.^{114,115} (ii) Dielectrophoresis for cell isolation is based on the motion of cells caused by polarization effects in a nonuniform electric field, depending on the electric properties of cells and surrounding media, cell size, cell shape, and frequency of the applied electric field.¹¹⁶ It was applied to cell separation and droplet sorting in microdevices at a very high sorting speed.^{117–119} (iii) Optical force, exploited in optical tweezers for cell trapping as mentioned previously, can also serve as a contactless switch to direct target cells to the sorting channel on microfluidic chips.^{120–122} (iv) Pulsed laser microbeam can induce cavitation bubbles in solution to drive small-volume

Fig. 5 Cell trap–release techniques. (A) Optical trap. Adapted from ref. 34 with permission of The Royal Society of Chemistry. (B) Dielectrophoresis (DEP) trap. Adapted from ref. 110 (Copyright (2013), with permission from Elsevier). (C) Optoelectronic tweezers (OET) trap. Adapted with permission from ref. 111. Copyright 2005 Nature Publishing Group.

Table 1 Comparison of active microfluidic cell isolation methods

Physics	Method	Mechanism	Main requirement	Throughput	Ref.
Electrics	Electrophoresis	Electrostatic actuation	Function generator, electrode	600 droplets per s	114,115
	Dielectrophoresis	Dielectrophoretic force	Function generator, electrode	30 000 droplets per s	116–119
Optics	Optical switching	Optical force	Laser source	20–100 cells per s	120–122
	Pulsed laser	Cavitation bubble	Pulsed laser system	6000 cells per s	123–125
Acoustics	Acoustophoresis	Acoustic radiation force	Function generator, electrode	3000 droplets per s	126–129
Mechanics	Piezoelectric actuator	Piezoelectric force	Piezoelectric actuator	1000 cells per s	130,131
	External valve	Pulsed pressure	Solenoid valve	30–50 cells per s	132,133

liquid jets for switching the cell flow in microchannels, and has been used for high-speed, high-purity, and high-viability cell sorting in a pulsed laser triggered fluorescence activated cell sorter.^{123–125} (v) Acoustophoresis for cell isolation is based on the movement of cells suspended in fluid and exposed to ultrasound fields driven by the acoustic radiation force. Effects of acoustophoresis on cells depend on size, density, and compressibility of the cell.¹²⁶ It has been demonstrated for on-chip sorting of cells, droplets, and microparticles.^{127–129} (vi) By directly using on-demand mechanical forces to perform cell isolation, piezoelectric actuator (PZT) with short response time (~ 0.1 ms) has been integrated for on-chip cell sorting, in which the PZT bending controlled by a voltage pulse can trigger a transverse displacement of fluid ($\sim$ subnanolitre volumes) for isolating target cells from the body flow.^{130,131} (vii) External-valve-based cell isolation methods hold several

advantages such as simple chip fabrication and easy platform integration. Not only can the external valve be used to switch the outer pressure source to push a small volume of fluid out of body flow for cell sorting,¹³² but a capillary-tuned external solenoid valve can directly provide a stable and controllable suction effect to realize cell/droplet sorting without any outer pressure sources.¹³³

4.3. Towards high-throughput microfluidic RACS

To achieve high-throughput microfluidic RACS, the above introduced techniques to improve quality and rate of SCRS acquisition as well as those to expedite cell isolation in a fast continuous mode should be coupled. A number of recent proof-of-concept experiments have been reported on this front.

As mentioned previously, microfluidics-based cell trap–release techniques can be introduced to ensure high efficiency

of SCRS acquisition by precisely aligning a fleeting cell with the Raman detection spot. Moreover, the cell trap–release strategies contribute to acquiring high-quality spontaneous Raman spectra of single cells in a continuous flow by elongating the temporal duration that is allowed for Raman acquisition, which is important for the implementation of continuous RACS, particularly when label-free, high-throughput RACS is still at its infancy. To date, a few cell trap–release based RACS platforms have been developed without the assistance of any enhanced Raman techniques. Among them, the optical tweezer (OT) based trap–release method appears the most readily to be integrated into microfluidic RACS, as the flow-based OT-RACS system developed by James W. Chan *et al.*,¹⁷ in which the laser simultaneously serves as the excitation source of Raman spectra, the trapping force, and the sorting switch. Cells could be trapped for Raman acquisition and the interested one then was dragged into the collection channel by the optical tweezers (Fig. 6A).¹⁷ Similarly, Jürgen Popp *et al.*^{20,34} proposed another OT-RACS system which integrated fiber laser for single-cell trapping and SCRS acquisition, and flow switching for target cell sorting on a quartz microfluidic chip (Fig. 6B). However the development of high-throughput OT-RACS platforms has been hampered by the slow processing

speed of optical tweezers. Faster cell trap–release methods, such as DEP, OET, *etc.*, are helpful to improve sorting throughput of RACS. Recently a novel DEP-RACS platform based on dielectrophoretic single-cell trap–release has been developed, which utilized a positive-DEP array for fast cell trap–release and combined with a solenoid-valve-suction-based switch for separation of individual cells (Fig. 6C).²¹ On this first prototype of high-throughput RACS, 52 yeast cells were sorted into the isolation channel within 9 min and the ratio of the carotenoid-producing yeast was elevated to 77% (original sample: 9%), representing 8-fold enrichment. It is expected that, with further improvement of cell trap–release and isolation rates or by utilizing high-sensitivity Raman techniques, RACS of much higher throughput can be achieved in the near future.

Several enhanced Raman technologies were reported to be integrated with flow cytometry for rapid SCRS acquisition. SERS flow cytometry was developed by John P. Nolan *et al.*,^{23,24,134,135} in which the Raman scattering from individual tags could be detected with submillisecond interrogation time (Fig. 7A). It has recently been shown to detect leukemia and lymphoma cells.²⁵ With improvement of reproducibility of SERS signals of cells by combining microdroplet techniques^{136–139} or advanced SERS substrates fabricated by self-assembly or nanofabrication methods,^{140–142} more reproducible and robust SERS signals should be produced for rapid SERS-activated cell sorting. In an RRS-activated cell counting platform, resonance Raman spectra of photosynthetic cells were acquired continuously 27 times per second (Fig. 7B).²⁶ Once further coupled with an isolation unit, RRS-activated cell sorting should be feasible on the microfluidic platform, which may be used to screen photosynthetic microorganisms based on sorting of pigment-containing cells. Benefiting from superb sensitivity and high imaging-speed (*e.g.*, at video rates), CARS and SRS have been successively utilized in label-free flow cytometry for the analysis of biological specimens (Fig. 7C).^{27,28,143,144} The rate of acquiring CARS spectra of flowing yeast cells could reach 100 Hz in the MCARS (multiplex coherent anti-Stokes Raman scattering) flow cytometry,²⁸ which shows excellent potential for high-throughput CARS- or SRS-activated cell sorting.

Furthermore, fast cell isolation techniques are a cornerstone of high-throughput RACS. With the recent improvement of SCRS acquisition speed, the speed of microfluidic cell isolation can become increasingly a bottleneck. The cell isolation techniques that have contributed to high-speed FACS platforms should potentially benefit high-throughput RACS as well. For instance, the pulsed laser used in FACS can achieve >80% sort purity and ~6000 cells per second sort throughput.¹²⁴ Feasibility of integrating pulsed laser into RACS has been proved in the recently reported Raman activated cell ejection (RACE) system.¹⁸ A microfluidics-based high-throughput RACS is expected to be realized, when the high-speed SCRS-based cell identification is combined with the pulsed laser triggered cell sorting. A recent study reported that fluorescence-activated droplet sorting by DEP could be performed at as fast as 30 kHz with >99% accuracy,¹⁴⁵ which indicates

Fig. 6 State-of-the-art of microfluidic RACS systems. (A) Optical tweezer-based RACS (OT-RACS) developed by James W. Chan *et al.* Adapted from ref. 17 with permission of The Royal Society of Chemistry. (B) Optical tweezer-based RACS (OT-RACS) developed by Jürgen Popp *et al.* Adapted from ref. 20 (with kind permission from Springer Science and Business Media). (C) Positive dielectrophoresis-based RACS (DEP-RACS). Adapted with permission from ref. 21. Copyright (2015) American Chemical Society.

Fig. 7 Several proof-of-concept developments on high-throughput microfluidics-based flow cytometry. (A) SERS-activated flow cytometry. Adapted with permission from ref. 134. Copyright (2009) American Chemical Society. (B) RRS-activated cell counting. Adapted with permission from ref. 26. Copyright (2012) American Chemical Society. (C) CARS-activated flow cytometry. Adapted with permission from ref. 27. Copyright (2008) Optical Society of America.

that ultra-high throughput RACS can potentially be obtained if such a droplet sorting method is introduced into an RACS system. Besides, sorting cell-encapsulating droplets instead of cells themselves not only enables much higher sorting rate, but avoids possible damage to the cell induced by the sorting force exerted. However, the risk is also apparent, as high Raman background of the oil for generating droplets may overwhelm the weak Raman signals of individual cells that are inside the droplets. A potential solution is to separately perform single cell Raman acquisition, single-cell droplet encapsulation, and sorting, in that SCRS-based cell identification is accomplished prior to the introduction of oil for droplet generation. Once this problem is resolved, the DEP-based droplet sorting method can be used in RACS to address the need for higher throughput.

5. Conclusion and prospects

The first generation of RACS systems that combine SCRS and micro-manipulation techniques for sorting cells of interested functions have been reported. However, compared with those widely used yet labeling-required or staining-dependent cell sorting technologies such as FACS and MACS, the weak Raman signal greatly limits the further development of the existing RACS systems to achieve high throughput in single-cell sorting. Strategies that can tackle this bottleneck are in two main categories: first, improvement of Raman-acquisition efficiency and quality based on advanced Raman spectrometers and enhanced Raman techniques; second, development of novel microfluidic devices for cell sorting followed by integration into a complete RACS system. Exploiting these strategies, prototypes for a new generation of RACS have been presented, such as flow-based OT-RACS, DEP-RACS, and SERS/CARS flow cytometry.

So far, these preliminary demonstrations of high-throughput RACS (HT-RACS) can reach an analytical rate of several to dozens of cells per second, which is already higher than the first generation of RACS (usually taking several minutes for each sorting event). But, there is still a long way to go to address the need of a real biological sample analysis. The biggest limitation remains the rate and efficiency of SCRS acquisition, though some of these RACS systems have been coupled with enhanced Raman techniques. Therefore, the realization of HT-RACS relies on a great improvement in SCRS acquisition. Besides, reliable synchronization of advanced Raman signal acquisition, identification and microfluidic cell sorting will greatly benefit the throughput of HT-RACS. It has been proved that the RRS detection time of photosynthetic cells can be as short as 1 ms, and thereby the theoretical throughput of RACS would be as high as kHz, but a real rate of only a few Hz was achieved in the RRS-activated cell counting system due to the delay of data recording, communication between different parts of the instrument, *etc.*²⁶ In addition, utilization of high-speed microfluidic cell isolation technologies such as pulsed laser based cell isolation and DEP based droplet sorting will definitely facilitate the accomplishment of HT-RACS with thousands of or much higher sorting throughput, which will certainly be able to compete with FACS. Eventually, such high-throughput microfluidic RACS will provide biologists with a powerful single-cell analysis tool to explore the scientific questions or applications that have been beyond the reach of FACS and MACS.

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