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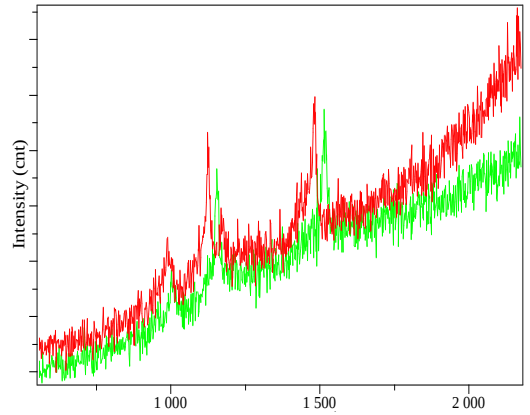
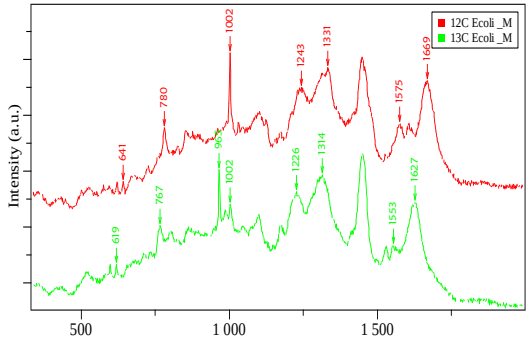
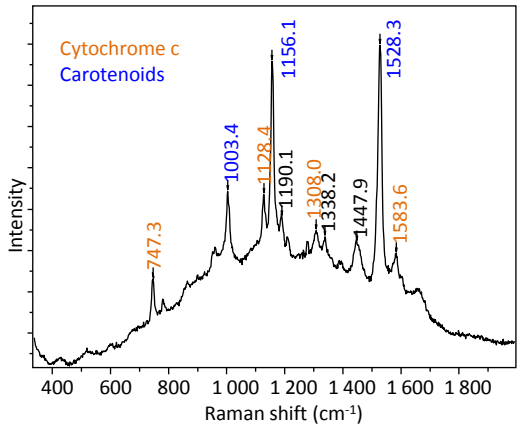
Abstract: Single cell Raman spectra (SCRS) are intrinsic biochemical profiles and 'chemical images' of single cells which can be used to characterise phenotypic changes, physiological states and functions of cells. On the base of SCRS, Raman activated cell sorting (RACS) provides a label-free cell sorting approach, which can link single cells to their chemical or phenotypic profiles. Overcoming naturally weak Raman signals, establishing Raman biomarker as sorting criteria to RACS and developing specific sorting technology are three challenges of developing RACS. Advances on Raman spectroscopy such as Stimulated Raman Scattering (SRS) and pre-screening helped to increase RACS sorting speed. Entire SCRS can be characterised using pattern recognition and specific Raman bands can be extracted as biomarkers for RACS. Recent advances on cell sorting technologies based on microfluidic device and surface-ejection enable accurate and reliable single cell sorting from complex samples. A high throughput RACS will be achievable in near future by integrating fast Raman detection system such as SRS with microfluidic RACS and Raman activated cell ejection (RACE).

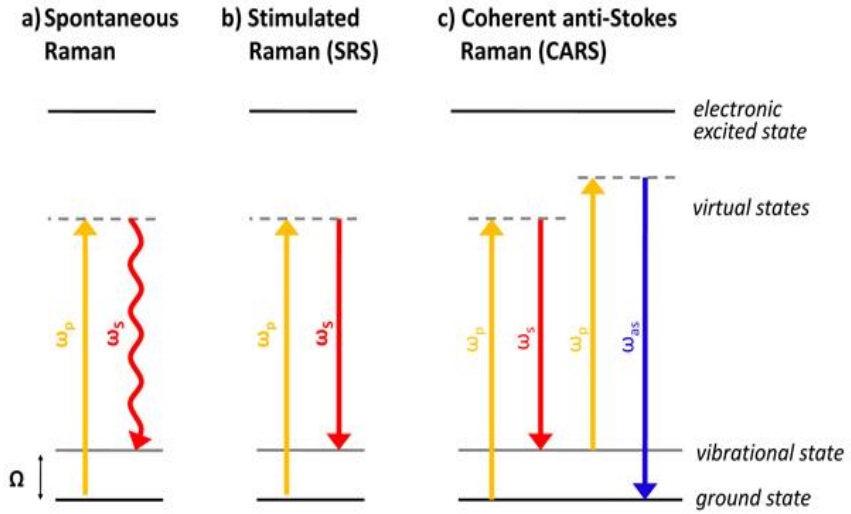
Graphical Abstract (for review)

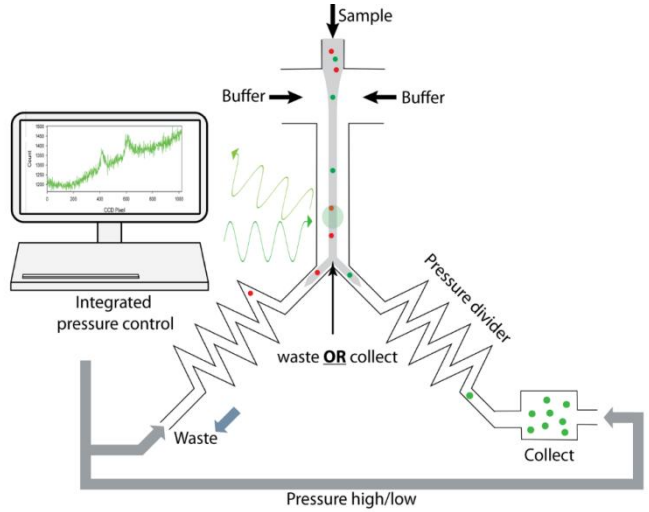
RACS sorting criteria → Raman signal enhancement → Cell sorting technology for RACS



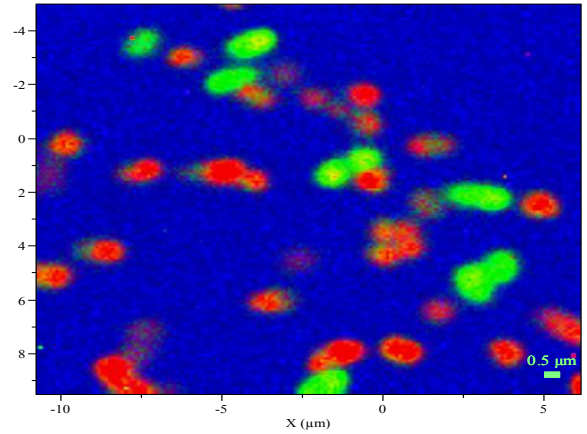








Microfluidic-RACS



Red: ^{12}C-bacteria green: ^{13}C-bacteria

Raman imaging mediated RACE

Raman activated cell sorting

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Highlights:

- Raman activated cell sorting (RACS) provides a label-free cell sorting approach, which can link single cells to their chemical or phenotypic profiles.
- There are three challenges for developing RACS: weak Raman signal, sorting criteria and specific sorting technology.
- Advances on Raman spectroscopy such as Stimulated Raman Scattering (SRS) and pre-screening will help increase RACS sorting speed. Entire single cell Raman spectra (SCRS) and specific Raman biomarkers can be used as sorting criteria for RACS. Recent advances on microfluidic and surface-ejection based cell sorting technologies enable accurate single cell sorting from complex samples.
- A high throughput RACS will be achievable in near future by integrating fast Raman detection system such as SRS with microfluidic RACS and Raman activated cell ejection (RACE).

Raman activated cell sorting

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Key Words: single cell sorting, Raman activated cell sorting (RACS), Raman activated cell ejection (RACE), stable isotope probing, Raman spectroscopy, metabolism, Raman biomarker.

Abstract:

Single cell Raman spectra (SCRS) are intrinsic biochemical profiles and ‘chemical images’ of single cells which can be used to characterise phenotypic changes, physiological states and functions of cells. On the base of SCRS, Raman activated cell sorting (RACS) provides a label-free cell sorting approach, which can link single cells to their chemical or phenotypic profiles. Overcoming naturally weak Raman signals, establishing Raman biomarker as sorting criteria to RACS and developing specific sorting technology are three challenges of developing RACS. Advances on Raman spectroscopy such as Stimulated Raman Scattering (SRS) and pre-screening helped to increase RACS sorting speed. Entire SCRS can be characterised using pattern recognition and specific Raman bands can be extracted as biomarkers for RACS. Recent advances on cell sorting technologies based on microfluidic device and surface-ejection enable accurate and reliable single cell sorting from complex samples. A high throughput RACS will be achievable in near future by integrating fast Raman detection system such as SRS with microfluidic RACS and Raman activated cell ejection (RACE).

Introduction

Single cells are the basic building blocks and functional units of all organisms on Earth. Cell individuality and phenotypic variation play important roles in many key areas of biology, including gene regulation and expression [1,2], stem cell differentiation and development [3,4], cancer [5] and function of microbial communities [6**]. Characterisation and linking genotype (e.g. single cell genomics/transcriptomics) and phenotype of single cells is central to modern biology [7,8].

Raman microspectroscopy is a label-free biochemical fingerprint technology, able to reveal intrinsic chemical information of individual cells, and this may be specific elicitation of gene expression, biosynthesis of compounds, cell components, characteristic structures, physiological states, or metabolic profiles [9-11].

Raman-activated cell sorting (RACS) is an emerging approach among the various single cell sorting techniques and its key advantage is no need of *external* labelling. A RACS system couples a detection instrument of single cell Raman spectra (SCRS) to a cell isolation system that can be operated in solution (i.e. Raman tweezers) [12**,13], in flow (microfluidic based RACS) [14**,15**] and on a surface (i.e. Raman activated cell ejection - RACE) [16*]. An illustration of three RACS systems is presented in Fig. 1A. Reviews on microfluidic technology and its application to Raman microscopy have been well documented [17**,18*].

In this review, we discuss the challenges and recent technology advances on RACS, and its application to microbiology and medical study.

To develop RACS, three challenges have to be addressed: weak Raman signal, sorting criteria and specific sorting technology. Spontaneous Raman scattering is naturally weak, as about 1 in 10^7 incident photons experience Raman scattering [19]. It usually requires long acquisition time (usually a few seconds) to obtain a SCRS, limiting the application of high throughput RACS. Since RACS sorts cells based on Raman spectra, Raman biomarkers are the key criteria. Unlike traditional fluorescence activated cell sorting (FACS) [20] which is based on fluorescent intensity in single cells, a SCRS usually contains more than a thousand of Raman bands (Table 1). Hence, identification and application of Raman biomarkers from SCRS for cell sorting can be complicated. RACS requires precise alignment of a single cell to the detection spot and reliable synchronisation of detection, on-the-fly classification and sorting processes. The sorting process in RACS has to be specially designed to hold cells for a few seconds in order to obtain Raman spectra from a single cell.

Naturally weak Raman signal

Instrument optimisation of Raman microspectroscopy can reduce acquisition time of spontaneous SCRS down to subseconds (0.1 s per cell) [16]. Resonance Raman scattering can detect cells containing Raman active molecules (e.g. carotenoids) at a rate of 1 ms per cell [21,22]. Although surface enhanced Raman scattering (SERS) can enhance Raman signals by a factor of 10^6 to 10^{14} [23], acquisition time of single cells by SERS is modestly 1 to 10 s [24*], which is still long for high throughput sorting. SERS selectively enhances some Raman bands and is related to nanoparticle sizes and wavelength of the incident laser. Furthermore, interpretation of spectra generated from SERS is also a challenge. These limit SERS application to RACS.

Coherent anti-Stokes Raman spectroscopy (CARS) [25] and Stimulated Raman scattering (SRS) [26**] overcome naturally weak signal of spontaneous Raman scattering [27], significantly shortening Raman acquisition time. SRS is specifically promising as it not only generates nearly identical spectra to spontaneous Raman spectroscopy, but also is quantitative and free of non-resonant noise [26]. SRS covers relatively small windows of molecular vibrations, whilst spontaneous Raman spectroscopy contains a broad range of vibrational spectrum. Hence, SRS and spontaneous Raman spectroscopy are complementary, as spontaneous Raman spectroscopy is useful to identify Raman biomarker and SRS offers speed advantages to sort cells. Current SRS can cover 200 wavenumbers with a speed of 32 μs per pixel [27**], which makes a high throughput RACS possible.

In addition to advances on high speed Raman instrumentation, another way to circumvent the problem of weak Raman signals is pre-screening which can selectively identify or enrich target cells before Raman measurement. Fluorescent *in-situ* hybridisation (FISH) can be used to sensitively and selectively pre-screen cells based on their phylogenetic identity (e.g. 16S-rRNA) [28]. Raman-FISH, a combination of FISH and Raman takes advantages of rapid fluorescent pre-screening and identification of cell metabolic activity by SCRS [16,29], enabling high throughput identification and sorting of metabolically active cells in complex samples [12**,30,31*]. Biocompatible magnetic nanoparticles (MNPs) were employed to pre-screen metabolically active and dividing cells from complex samples [31]. Initially all cells in biosludge were coated with MNPs and then re-introduced into the original wastewater. Cells that were metabolically active and dividing lost MNPs and became free, whilst the inactive and non-dividing cells remained MNP-coated, immobilised by a permanent magnet [31]. Raman measurement confirmed that this MNP-mediated pre-screen method contained

79% of target cells enriched from a complex biosludge [31]. Antibodies can also be used to enrich cells of interest. Raman biocompatible aluminium (Al) surface can be attached with antibodies through organosilane (3-glycidyloxypropyl) trimethoxysilane (GOPS). The antibody modified Al surfaces were designed to specifically target lipopolysaccharides for Gram-negative bacteria and cell wall lipoteichoic for Gram-positive bacteria, and importantly the coating has no interference on Raman spectral acquisition [32].

Raman biomarker as sorting criteria

A SCRS is the sum of all molecular vibrational profile from a single cell, which contains many overlapped Raman moieties. RACS can sort cells based on native and stable-isotope labelled SCRS through stable isotope probing (SIP) (Fig. 1B and 1C).

The entire Raman spectra and specific Raman bands have been used as sorting criteria for RACS (Fig.1B). Multivariate data analysis and pattern recognition tools such as principal component analysis (PCA) [33,34], discriminant analysis (DFA) [16], artificial neural network (ANN) [35] and machine learning [36] can be used to sort cells based on whole SCRS patterns. Relative intensity of some Raman bands can be used to discriminate cell types. For example, Raman bands of tryptophan-rich protein at 752 cm^{-1} and nucleic acids at 785 cm^{-1} were used to distinguish different human cells (keratinocytes and fibroblasts) and to identify tumorigenic cells from the two types [37]. Raman bands corresponding to specific compounds in cells can also be used as Raman biomarkers, for example polyphosphate [38], glycogen [39], polyhydroxybutyrate (PHB) [40], cytochrome c [41,42], cell pigments [43] (e.g. carotenoids [22], chlorophyll [44]), calcium dipicolinate (CaDPA) [45], polysulfide and cyclooctasulfur [42], starch [46], triacylglycerol (TAG) [34]. A reference Raman spectral database of biological molecules has been documented in Gelder et al., [47] and a summary of Raman band assignments to biological molecules has been provided in supplementary Table S1 in a recent review [48*]. However, due to the complexity of cell and infancy of Raman application to the study of single cells, many existing and new Raman bands in SCRS are not properly assigned yet. Hence, comprehensive assignment to biological molecules and identification of Raman biomarkers are needed in the future.

Some Raman bands of SCRS shift when the cells incorporate stable isotope atoms such as ^{13}C, ^{15}N and ^{2}H [16,22,29] (Fig. 1C). SIP-SCRS is able to establish a link between substrate metabolism (e.g. 13-C and 15-N labelled substrates) and the corresponding cells at single cell level [16,31]. In addition, a universal Raman biomarker can be applied to probe general

activity of cells in a complex microbial community by simply adding heavy water (D_2O) into cell cultures [12**]. In the presence of D_2O, metabolically active cells will incorporate the deuterium from D_2O into the cells via NADH/NADPH regeneration and the newly formed carbon-deuterium (C-D) bond has distinct vibration mode shifted from the C-H bond [12**]. These cells could be unambiguously detected via their SCRS by identifying C-D signature peaks appearing in the region between 2040 and 2300 cm^{-1} that typically has no detectable peaks in the SCRS of non-deuterium-labelled cells (Fig. 1C). A review about application of SIP-Raman to microbiology is in press [48*].

Raman Activated Cell Sorting: development and state-of-the-art

Fundamental to RACS is the capability of synchronising four basic operations: 1) locating a sample in the detection point, 2) acquisition of sufficient Raman signals of a cell, 3) rapid *in-situ* analysis and on-the-fly decision making, and 4) triggering the isolation of the target sample into the collection. Although each function is indispensable for an effective RACS system, the strength of the Raman signals from a sample is the dominant factor limiting the development of the system. Table 1 summarises three RACS technologies and compares them with FACS. RACS is slow sorting technology in comparison with FACS, because of the weak spontaneous Raman scattering. The majority of RACS were based on trapping and release process until recent trapping free RACS has been developed [15]. The sorting systems in RACS can be largely categorised as RACS *in solution, in flow* and *on surface*, as discussed below.

RACS in Solution: Raman sorting coupled with cell physical trapping

Optical tweezers employ a highly focused laser beam to provide an attractive or repulsive force to physically hold and move microscopic, neutral objects [49]. Progress has been made for label-free detection and discrimination of individual cells using Raman tweezers that couples Raman microspectroscopy to optical tweezers [13,50-52].

Huang et al has proven the concept of Raman optical tweezers cell sorting by applying Raman tweezers to identify and sort cells in capillary tubes [13]. Recently, by applying D_2O to mouse cecal microbiota, metabolically active cells stimulated by mucin and glucosamine were identified and sorted by Raman tweezers [12**]. From each sample, 40 cells from cecum with high C-D bands were manually moved to the sterilised end of the capillary tubes. The cells were then harvested in an eppendorf tube and their genomes were amplified and subsequently sequenced [12**]. Although the Raman tweezers process is slow due to the

small size and vulnerability of cells, this work proved that manually operated Raman tweezers with assistance of Raman-FISH pre-screening can be applied to complex samples [12**].

Raman tweezers is suitable for the study of cells in water (e.g. river and sea water) as the cells are trapped and analysed in their native condition. However, single cell trapping by laser and moving the cell along for sorting would lead to a long time exposure time to cells. In the original report, it took averagely three minutes for each cell to be measured and sorted [13]. Relating to this, one issue that needs to be addressed is the laser damage effect on the cell, especially for phototrophs whose reaction centres are sensitive to photodamage. The photodamage effect is wavelength dependent. It was found that radiation at 735, 785, 835 nm irreversibly suppressed the photochemical activity of microalga *Trachydiscus* sp. at a power of 25 mW, whilst the 885, 935 and 1064 nm laser had no adverse effect [53]. Lasers also have detrimental impact on non-phototrophic cells. For example, 1064 nm laser tweezer with the energy of 0.36 J affected *E. coli* cell division and an energy of 0.54 J affected cell growth [54].

To increase cell sorting rate, Raman tweezers were integrated with a microfluidic chip [52, 55]. Cells travelled through a channel on the microfluidic, and were trapped by a 1064 nm laser. A continuous 532 nm laser was then used to generate Raman scattering and acquire spectra at 1 second intervals [52]. Application of Raman tweezers in microfluidic device enables single cells to be optically trapped and analysed by Raman spectroscopy in an automated fashion [52].

RACS in flow: towards continuous, automated Raman sorting

Sorting cells in solution offers great advantages in maintaining cell viability. Furthermore, advanced cell handing in solution, such as microfluidic technologies, offers great scope in realizing the whole RACS process in flow on a single device. Since hydrodynamic forces are generated in a flow and in proportion to the flow velocity, stronger forces are needed to trap cells in a flow during Raman acquisition. A range types of force actuators can be used for cell trapping, such as optical, mechanical, magnetic, dielectrophoretic, electrophoretic, and acoustic forces [56]. Dielectrophoresis (DEP) is an effect where a polarizable particle will move in a non-uniform electric field. The electric field causes a polarization of the particle, which then will experience an attractive or repulsive force towards regions of larger field intensity [57]. DEP has been employed to trap bacteria in dilute suspensions for obtaining

high quality Raman spectra with an integration time of only one second. *E. coli* and *Enterococcus faecalis* can be classified within a few minutes [58].

Zhang et al. presented an alternative pause-and-sort RACS microfluidic system that combines positive dielectrophoresis (pDEP) for single-cell trapping and release with a solenoid-valve-suction-based switch for cell separation. This has allowed the integration of trapping, Raman identification, and automatic separation of individual cells in a high-speed flow. By exerting a periodical pDEP field, single cells were trapped, ordered, and positioned individually to the detection point for Raman measurement. As a proof-of-concept demonstration, a mixture of two cell strains containing carotenoid-producing yeast (9%) and non-carotenoid-producing *Saccharomyces cerevisiae* (91%) was sorted, which enriched the former to 73% on average and showed fast Raman-activated cell sorting at the subsecond level [14]. However, DEP trapping forces strongly depend on medium conductivity (normally requiring low conductivity) and sample sizes, which can compromise DEP-RACS sorting of the systems containing complex compositions or physiological ionic strength.

It is only recently the first trap-free RACS in a flow has been reported, enabling continuous and automated sorting of individual cells based on intrinsic Raman signals [15]. In contrast to trapping or immobilisation dependent approaches, this system is capable of reliable Raman acquisition of moving cells (from 100s mini-seconds to ~1 s). This does not depend on the physical properties of samples and medium, and offers unique advantages of sorting cells from a complex community and in their native environments (e.g. real-world sea water sample).

The system is capable of using simple hydrodynamic focusing and a pressure switch mechanism to sort individual cells based on intrinsic Raman signals [15] (Fig. 2). Key to this development is the implementation of integrated microfluidic pressure dividers. It eliminates flow fluctuations in the detection region, and maintains reliable Raman acquisition regardless of pressure variations elsewhere in the system. In addition, fast on-the-fly classification of cells was achieved via programmed, multi-parameter analysis of Raman spectra. The whole sorting process was seamless synchronised through integration of both hardware and on-line signal processing software. Using a model strain of cyanobacteria, a high accuracy sorting of 96.3% and a sorting frequency of 2 Hz were demonstrated. Higher throughput is possible with the realisation of reduced actuation delay through improved hardware (e.g. CCD camera and pump).

On surface: Raman activated cell ejection (RACE)

Both Raman optical tweezers and microfluidic-RACS require the cell to be suspended in solution. However, in many real-world samples, cells are distributed in biofilms, sediments, soils, human/animal feces and tissues. It is difficult to use microfluidic-RACS sorting cells from those complex samples because large particles or debris would easily block the nozzle and channels in microfluidic devices. In some cases, it is important to maintain spatial organisation of cells in their native environment to understand their interactions and functions. Hence, Raman activated cell ejection (RACE) approach has been developed to isolate cells of interest in their biological niches.

Although the concept of RACE was reported in 2013 [16], it used two separate instruments. In this study, initially SCRS from the cells mounted on the CaF_2 slide were obtained in Raman micro-spectroscopy, subsequently the slide was transferred to a laser microdissection microsystem for cell sorting [16]. A Zeiss PALM MicroBeam equipped with 337 nm pulsed laser was employed to isolate single cells by Laser induced forward transfer (LIFT) [16]. In that study, the thin water layer around cells evaporated after absorbing the pulsed-laser energy, and provided forward momentum to push the cells off the slide into a collecting tube lid [16]. However, this approach involves two operations of two separate systems, and a high power UV laser usually disintegrates cells, which hindered its application in Raman sorting and associated single cell genomics.

The first all-in-one system to perform RACE has recently been developed by Song and co-workers [59**] (paper under review). In this approach, cells were mounted onto a chip with a thin Al layer. The Al coating contributed no Raman signal to SCRS, provided better optical imaging of cells, and increased the stability of LIFT. The pulsed laser power in this system was 1000 times lower than other LIFT systems, significantly reducing potential photo- and thermal- damage to cells. Using the all-in-one RACE technique, potential phototrophs in the Red Sea sample were identified using carotenoids Raman signals as biomarkers. Single cells were ejected into a collecting well and whole genome amplification was performed on chip (Fig. 3). This is the first demonstration that applies RACE coupled single cell genomics to a real environment sample [59**].

Conclusion and outlooks

RACS as a label-free sorting technology will open a new frontier for cell biology to understand phenotypic heterogeneity in isogenic cell population, probe single cell functions in complex community and explore uncultured bacteria in nature. Armed with advanced

Raman spectroscopy and novel sorting technologies, RACS is expected to make significant progress in the future. A high throughput RACS will be achievable by integrating fast Raman detection system such as SRS with microfluidic RACS and RACE.

Acknowledgements

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Figures

Figure 1. (a) Three techniques that could realize Raman activated cell sorting (RACS), including Raman tweezers-RACS [13] , microfluidic-RACS [14], and RACE [59]. (b) The native Raman signal used as biomarkers in single cell sorting, including whole spectra [61] or specific bands [22]. (C) The stable isotope labelling (13-C [13] and 2-H [12]) generates Raman shift as biomarker.

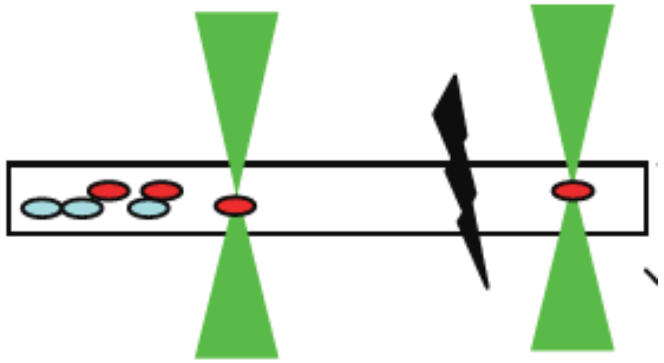
Figure 2. Automated, trap-free RACS in flow. This approach utilises simple hydrodynamic focusing and a pressure switch mechanism to sort individual cells based on intrinsic Raman signals [15].

Figure 3. An illustration of how RACE worked to isolate cells of interest [62]. (a) Bacteria were mounted on to a specific slide coated with laser absorbing material. (b) Microscopic image of bacteria on the slide. (c) The cells circled in (b) was found to be 13C-cells according to SCRS. (d) and (e) The cell of interest was ejected using LIFT. (f) The genome of the isolated cell was amplified using single-cell multiple displacement amplification.

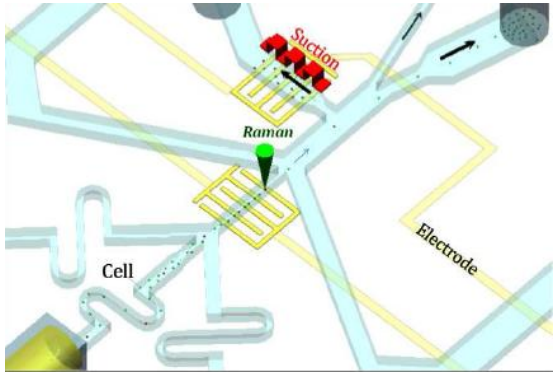
Figures
Raman activated cell sorting

Yizhi Song, Huabing Yin and Wei E. Huang

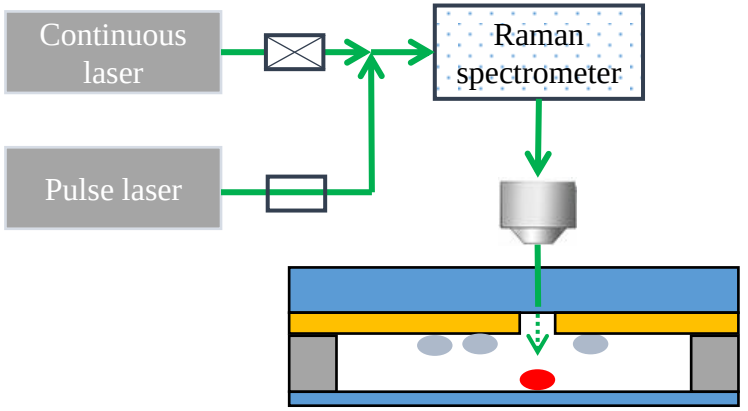
(a)



Raman tweezer-RACS



microfluidic-RACS



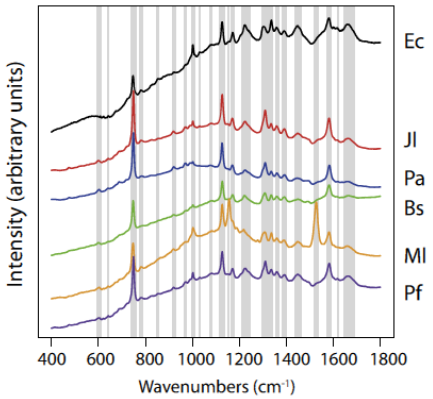
RACE

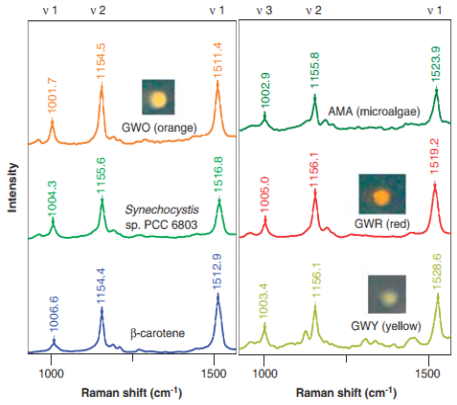
(b)

Native signal

Whole spectra

Bands correlates to specific compounds



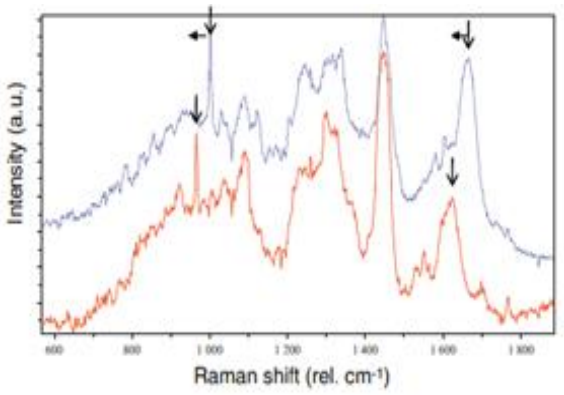


(c)

Stable isotope labelling

13 C labelling

Deuterium labelling



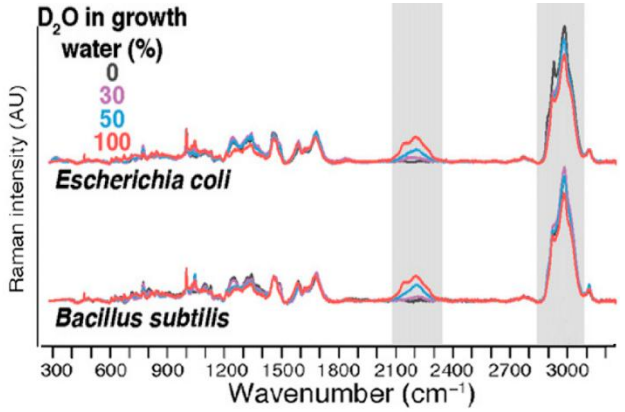


Figure 1

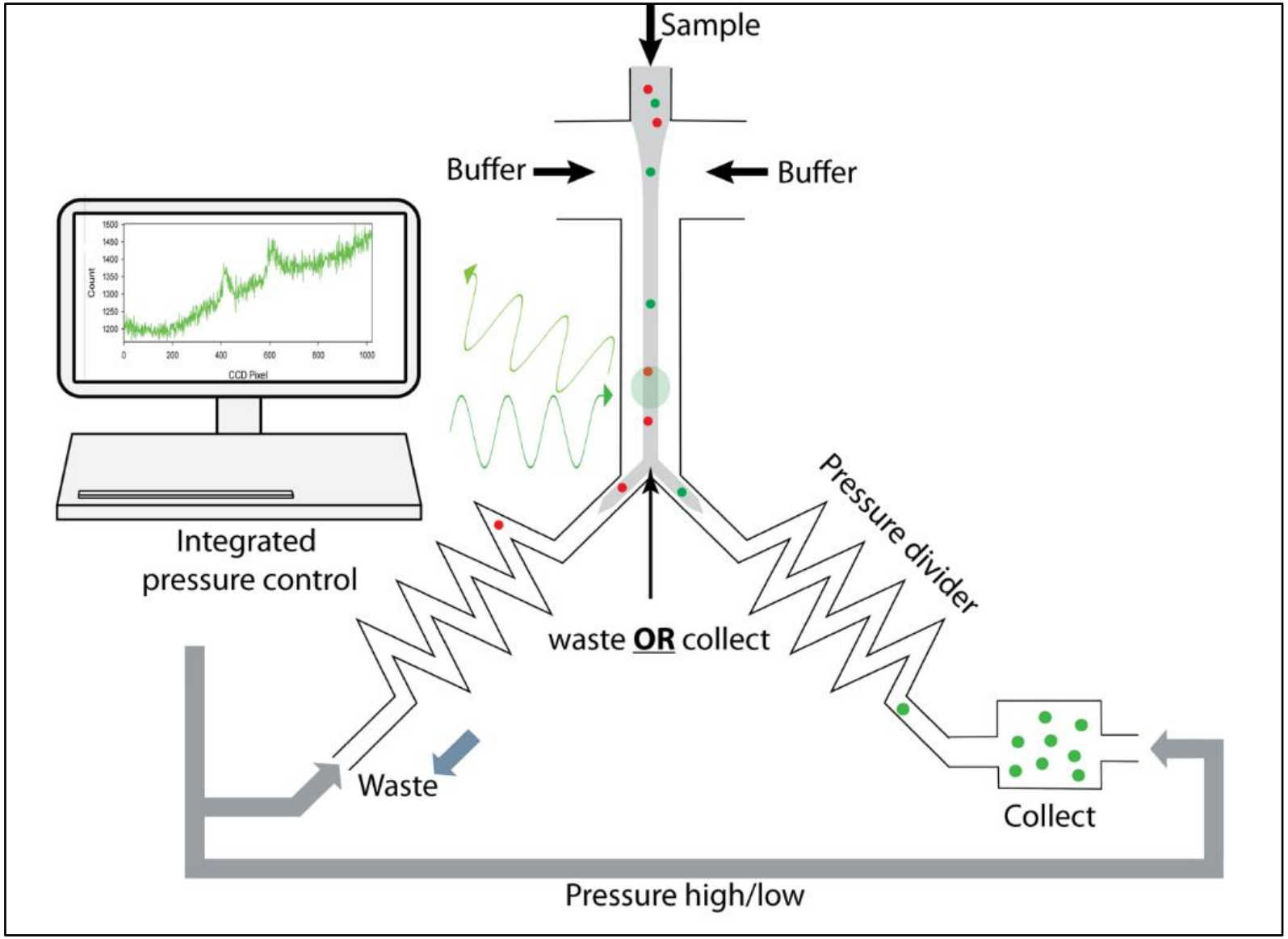

Sample
Buffer
Buffer
Count
CCD Pixel
Integrated
pressure control
Pressure divider
waste OR collect
Waste
Collect
Pressure high/low

Figure 2

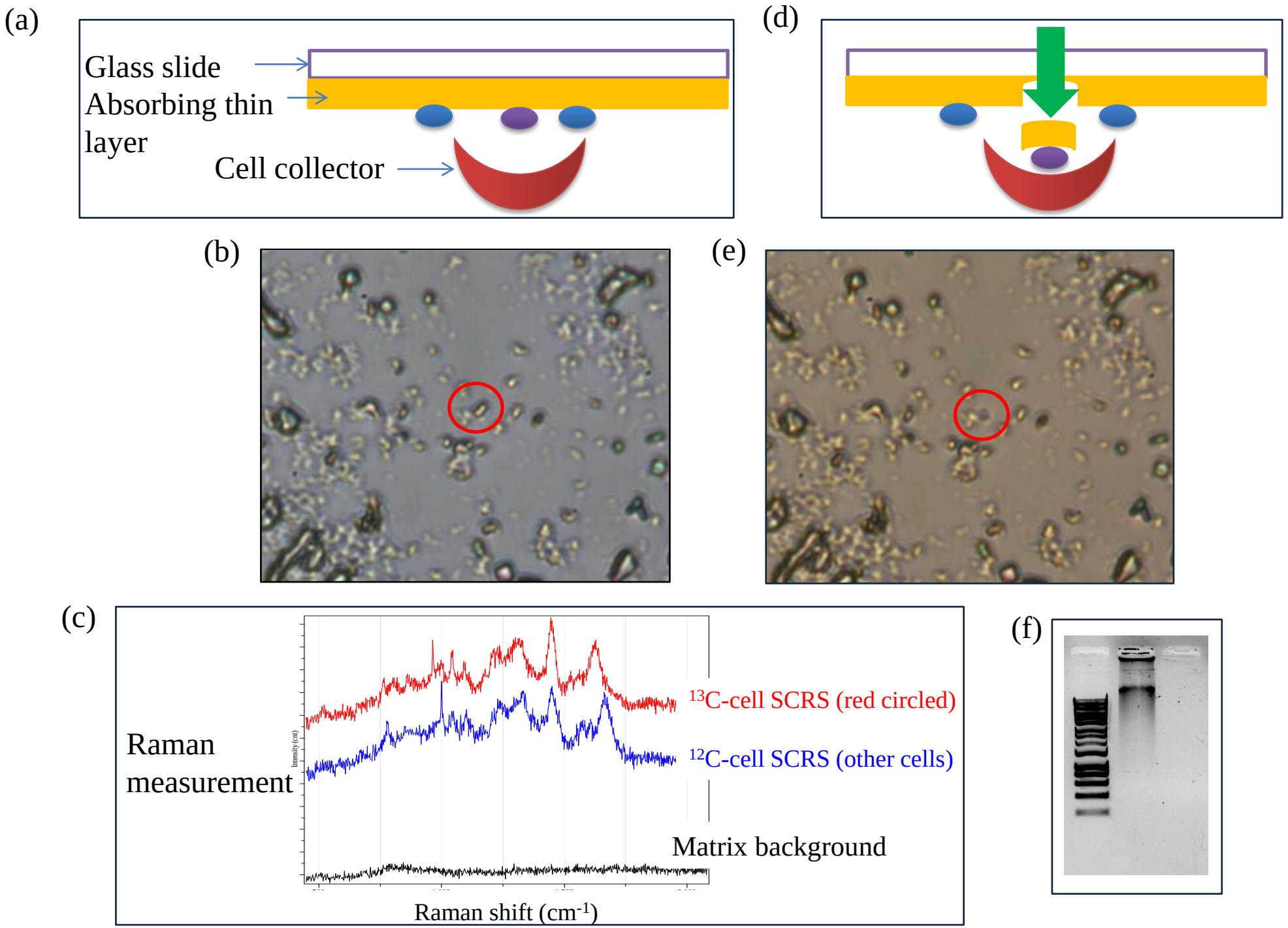

(a)
Glass slide
Absorbing thin layer
Cell collector
(d)
(b)
(e)
(c)
Raman measurement
Intensity (cnt)
^{13}C-cell SCRS (red circled)
^{12}C-cell SCRS (other cells)
Matrix background
Raman shift (cm^{-1})
(f)

Figure 3

Table 1: Comparison of fluorescent activated cell sorting and different Raman activated cell sorting techniques

	FACS	**Raman tweezer-RACS**	**microfluidic -RACS**	**RACE**
Labelling and selection criteria	*Externally* fluorescent labelling[1]. Pre-knowledge required for labelling	*Externally* label free[2] Intrinsic fingerprint or stable isotope labelling. No pre-knowledge required.		
Amount of the information	About 17 fluorescent colours and 2 physical parameters	Thousands of data regarding the biochemical and metabolic features of the cell, such as nucleic acids, protein, carbohydrate and lipids.		
Damage to the cell	Invasive or non-invasive[3]	Non-invasive		
Sample condition	Cells in suspension. Difficult to do in-*vivo*	Cells in suspension. In-*vivo* possible	Cells in suspension. in-*vivo* difficult	Cells in suspension, tissue or attached to solid surface. in-*vivo* possible
Sorting time	Up to 5000 cell/ second	~3 mins / cell	5 – 100 cell / second	1 cell /second
Contamination issue	Complicated system to ensure sterile	Relatively easy to maintain sterile	Complicated system to ensure sterile	Relatively easy to maintain sterile
reference	[60]	[12,13]	[14,15,22]	[59]

Note: 1. No externally fluorescent labelling to naturally fluorescent samples or expression of fluorescent protein in single cells. 2. Sometimes use stable isotope labelling which is internally labelled. 3. Non-invasive to naturally fluorescent samples or expression of fluorescent protein in single cells.