TUTORIAL REVIEW

Check for updates

Fluorescent analysis of bioactive molecules in single cells based on microfluidic chips

Yuanyuan Fan,† Defang Dong,† Qingling Li, Haibin Si, Haimeng Pei, Lu Li* and Bo Tang (iD)*

Single-cell analysis of bioactive molecules is an essential strategy for a better understanding of cell biology, exploring cell heterogeneity, and improvement of the ability to detect early diseases. In single-cell analysis, highly efficient single-cell manipulation techniques and high-sensitive detection schemes are in urgent need. The rapid development of fluorescent analysis techniques combined with microfluidic chips have offered a widely applicable solution. Thus, in this review, we mainly focus on the application of fluorescence methods in components analysis on microchips at a single-cell level. By targeting different types of biological molecules in cells such as nucleic acids, proteins, and active small molecules, we specially introduce and comment on their corresponding fluorescent probes, fluorescence labelling and sensing strategies, and different fluorescence detection instruments used in single-cell analysis on a microfluidic chip. We hope that through this review, readers will have a better understanding of single-cell fluorescence analysis, especially for single-cell component fluorescence analysis based on microfluidic chips.

1. Introduction

Cells are the most basic entities for an organism's physiological activity.[1] Even under the same physiological conditions, cells of the same type may show heterogeneity in their expression of various biological molecules. When cells are exposed

College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Institute of Molecular and Nano Science, Shandong Normal University, Jinan 250014, P. R. China. E-mail: tangb@sdnu.edu.cn, lilu5252@163.com
† Authors contributed equally.

to external stimulus or under a diseased condition, obvious differences of intracellular molecules expression could also be observed, which contributed to various destinies of cells.[2,3] Traditional analytical methods for biomolecule's detections are suitable for common bulk cell assays. However, the average data obtained would conceal the differences in molecular expression among individual cells, causing loss of important biological information. Thus, the detection and analysis of cellular components at the single-cell level is not only the essential way to demonstrate cell heterogeneity and differentiation, but also an effective means to accurately study the relationships of multiple molecules and their

Yuanyuan Fan is currently pursuing a Ph.D. degree at the college of Chemistry, Chemical Engineering and Materials Science in Shandong Normal University. She received her master's degree in analysis chemistry in 2017 from Shandong Normal University. Her research interests are focused on single-cell analysis of biomolecules on microfluidic chips.

Yuanyuan Fan

Dr Lu Li is currently a professor at the college of Chemistry, Chemical Engineering and Materials Science in Shandong Normal University. She received her Ph.D. in analysis chemistry in 2010 from Shandong University. Her research interests include single-cell analysis, single-molecule detection and fluorescence imaging of intracellular biomolecules.

Lu Li

related signalling pathways in cells.[2,4,5] Compared with cell homogenate samples in groups of cell assays, single cell samples have many unique characters.[6] For example, most types of cells have small volumes of only 0.1–8 pL. Common sample preparation and detection methods are not suitable for single-cell manipulation.[7] In addition, many of the intracellular components are in low copy numbers in single cells so highly sensitive detection schemes are in urgent need. Therefore, single-cell analysis presents a variety of challenges for any sample treatment and detection method. Critical issues to be resolved include how to perform highly efficient single-cell manipulations and conduct highly sensitive signal sensing and detection.

Microfluidic chips have shown great ability with accurate fluid control, cell manipulation, and signal output, which has proven to be a favourable tool for rapid and high-through single-cell analysis.[8–10] First, the microfluidic chip is a flexible platform, as its structure and function can be designed according to demands of single-cell analysis plus fluid control and cell samples control can be conducted by various modes such as hydraulic pressure, electric field, micro-valve and micro-dam structure, and oil–water interface interaction (microdroplets formation, fusion and separation).[11–14] Second, the tiny space in a microchip may strongly inhibit diffusion and consumption of trace amounts of cellular samples, improving their chemical or biological reaction rates and enriching the products to obtain higher signals.[15] Moreover, microfluidic chips also have open features and multiple detection techniques such as optics, electricity, magnetics *etc.*, can be easily integrated. For example, electrochemical detection of single cells has been performed on microchips by integrated microelectrodes on microchannels and reservoirs.[16] Combined with magnetic materials, cells or cellular components can be operated and detected in a magnetic field.[17,18] Optical technologies including fluorescence detection, surface enhanced Raman spectroscopy (SERS), surface plasmon resonance (SPR), and interferometry have been widely employed with microfluidic chips to make single-cell analysis.[19,20] Due to the unique advantages of microfluidic chips, various methods based on them have been developed for single-cell analysis. Many reviews have introduced single-cell manipulation, single-cell sample treatment, and various detection methods from a microchips standpoint, which have revealed a broad visual field for us of single-cell analysis based on microfluidic chips.[9,10,21,22]

The fluorescence technique has attracted tremendous attention by related researches in analytical chemistry, cell biology, design and development of analytical instruments, since it has several advantages such as high sensitivity, reproducibility, simplicity, and is easy to integrate.[23–25] It is also highly suitable for cellular components detection. In the field of single-cell analysis based on microfluidic chips, quite a few research areas are relevant to fluorescence methods for cellular components detection.[26,27] We think the main reasons are as follows: 1, the fluorescence technique allows non-invasive detection and, when integrated with microfluidic chips, there are no special processing requirements with the chips. 2, Various fluorescence probes, which can specially recognize cellular components, have been developed based on different molecular recognition mechanisms and signal sensing methods.[28,29] These make fluorescence methods widely applicable for kinds of biomolecules detection with high specificity, plus multiple kinds of molecules in individual cells can be simultaneously detected. 3, With improvements of fluorescence methods and instruments, the sensitivity of fluorescence detection can fully meet the needs of single-cell analysis, even reaching to the single-molecule level.[27] Moreover, different fluorescence methods would supply multiple information about concentration, dynamics, and distribution of cellular bioactive molecules, as well as relationships or heterogeneity among different cells.[15,30,31] Overall, fluorescence analysis on a microfluidic chip possesses the ability of highly efficient sample manipulation with a microfluidic chip, the ability of specific recognition and multicomponent labelling and sensing based on different kinds of fluorescent probes, and the ability of highly sensitive and multi-parameter detection by different fluorescence methods and instruments. These characters make this technique very promising and advantageous for single-cell component analysis.

However, many reviews have comprehensively introduced various functions and applications of microfluidic chips in single-cell manipulations, processing, and analysis. Besides, a lot of articles have reviewed different detection methods and instruments used in single-cell analysis.[32–34] Therefore, in this review, we will pay less attention to the design and function of chips, and instead mainly focus on the application of fluorescence methods in components analysis on microchips at the single-cell level. By targeting different types of biological molecules in cells such as nucleic acids, proteins, and active small molecules, we will specially introduce and comment on their corresponding fluorescent probes, fluorescence labelling and sensing strategies, and different

Bo Tang obtained his PhD in analytical chemistry in 1994 from Nankai University. Then he joined the College of Chemistry, Chemical Engineering and Materials Science as a full professor at Shandong Normal University. His current research interests include development of molecular and nano probes for analytical and biomedical applications, solar energy chemical transformation and storage, and clean synthesis of chemicals. He has more than 400 papers published in peer-reviewed journals and his work has been cited in excess of 10 000 times (2017).

Bo Tang

fluorescence detection instruments used with microfluidic chips. It is hoped that through this review, readers will have a better understanding of single-cell fluorescence analysis, especially for single-cell component fluorescence analysis based on microfluidic chips.

2. Fluorescence labelling and sensing for single-cell components analysis on microchips

2.1 Fluorescence labelling and sensing of nucleic acids

Nucleic acids, polymerized by many nucleotides, are essential for all forms of life.[35,36] According to their different chemical compositions, nucleic acids can be divided into ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) groups. DNA provides an essential foundation for genetic information storage, replication, and transfer in living cells.[37–39] RNA plays a key role in protein biosynthesis. During the synthesis process, messenger RNAs (mRNAs) are used as the template and amino acids are transported by transfer RNAs (tRNAs). Ribosomal RNA (rRNA) and ribosomal proteins are assembled to make a functional ribosome, which catalyses the sequential addition of amino acids to a growing polypeptide chain.[40–43] In addition, microRNAs (miRNAs) are endogenous ~22-nucleotide RNAs, which can regulate gene expression by targeting mRNAs for translational repression.[44–46] Nucleic acids are composed of paired organic bases with different arrangements and diversity in sequence length and base sequence. Biologically originated nucleic acids usually have relatively low quantities in living cells.[47] Therefore, specific recognition and signal amplification are two key issues for single-cell nucleic acids analysis. In a general way, the identification of nucleic acid molecules is based on a specific complementary hybridization reaction between target nucleic acid strands and probe strands. The sequence of the probe strands can be designed according to the target sequence.[48,49] For example, 16 s rRNA in single *E. coli* cells could be directly identified by peptide nucleic acid (PNA) probes with a complementary sequence and then fluorescent sensed by fluorescence resonance energy transfer (FRET).[50] To improve the sensitivity of nucleic acids detection in single cells, a variety of DNA amplification techniques has been applied, such polymerase chain reaction (PCR), rolling circle amplification (RCA), strand displacement amplification (SDA), hybridization chain reaction (HCR), and so on.[51–55] Among them, PCR was the most popular technology for the nucleic acid amplification and detection within single cells.[36]

2.1.1 PCR for single-cell nucleic acids analysis. PCR was proposed in 1985 by Mullis, and it was used for specifically amplifying nucleic acid *in vitro*.[56] It has become one of the most common and important molecular biological techniques ever since it emerged because of its high sensitivity, specificity, and swiftness. Single-cell PCR usually includes single-cell isolation, cell lysis, DNA amplification, and sequencing. Due to the convenience and accuracy of micro-

fluidic chips for single-cell manipulation, the above processes can be partially or completely carried out on a microfluidic chip to improve analytical performance. For instance, Hui Tian *et al.* utilized ligation-based droplet digital polymerase chain reaction (dd-PCR) for single-cell miRNA analysis (Fig. 1A).[57] After lysis of single cells, the released target miRNAs could be combined with partially complementary probes A and B and then T4 RNA ligase 2 would catalysis the ligation of probes A and B. The ligation products together with the PCR mixture were encapsulated in water-in-oil droplets and PCR amplification was triggered using the ligated DNA as a template. The quantified detection of miRNAs in a single cell could be accomplished by counting the number of fluorescence-positive droplets. Additionally, single-cell gene expression profiling based on PCR is a common method for analysis of circulating tumor cells. For the extremely low abundance of circulating tumor cells (1–3000 CTCs per mL) in the blood,[58] microfluidic technology has served as a high-efficiency platform for the enrichment of CTCs from blood samples and the gene expression of obtained CTCs could be analyzed subsequently. In this field, Toner *et al.* have done a lot of research. They exploited a CTC-iChip[59] (Fig. 1B(a)) for efficient sorting of CTCs from blood and performed a series of studies on the genes expression in a single CTC. For example, Maheswaran and Haber *et al.*[60] acquired 77 CTCs from 13 prostate cancer patients with drug resistance, and then analyzed the gene's expression in isolated single prostate CTCs using single-cell RNA-sequencing (RNA-Seq) technology. The results showed considerable heterogeneity from each individual cell and relevance of androgen receptor inhibitor and Wnt signaling pathways. Subsequently, this group researched the expression of extracellular matrix genes in mouse and human pancreatic CTCs by this method (Fig. 1B(b)).[61] Results revealed that extracellular matrix genes are highly expressed in CTCs and the extracellular matrix protein SPARC was closely related to the metastasis of pancreatic tumor. All of the above single-cell gene analysis works were carried out partially on microfluidic chips. A. K. White *et al.*[62] developed an integrated a microfluidic device that could execute all steps of single-cell capture, lysis, reverse transcription, and qPCR (Fig. 1C). The microchip could be divided into four main portions: (i) single-cell capture chamber, (ii) single-cell lysis chamber, (iii) RT chamber for reverse transcription, and (iv) 50 nL PCR chamber for amplification and detection. Using a TaqMan probe as indicator, multiple miRNA and mRNA in single embryonic stem cells were studied with this microchip. Subsequently, the author also measured the expression of miR-16, EEF2K, BCR-ABL transcripts, and GAPDH mRNA transcripts in single K562 cells on a similar microchip with the same detection process (Fig. 1D).[63]

Previous research showed that cell lysate had an inhibition effect on RT-PCR; thus, it is considered to be important for researchers to understand how to overcome the lysate-mediated inhibition of RT-PCR.[64] Dennis J. Eastburn *et al.*[65] applied an electric field to merger drops thereby increasing the drop volume and dilute cell lysate concentration
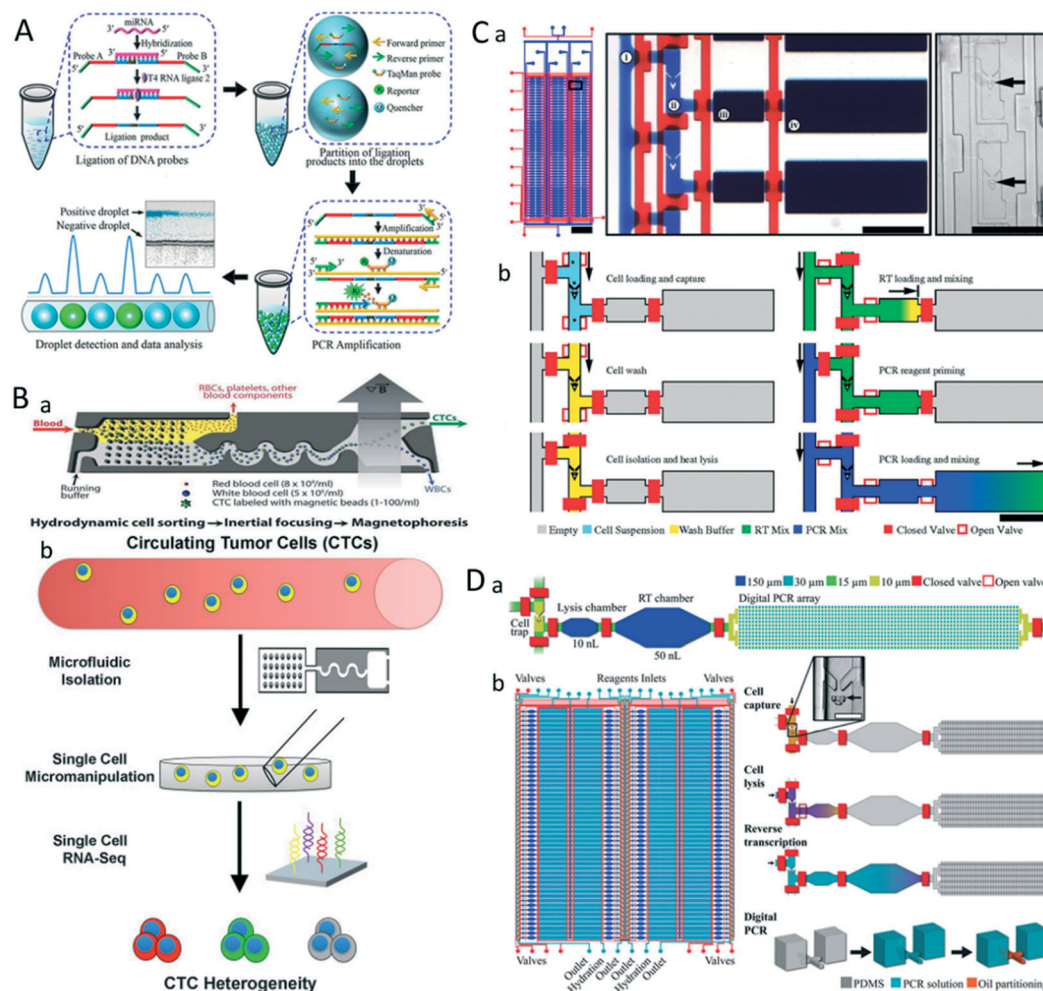
Fig. 1 A) The miRNA detection schematic based on droplet digital-PCR (reprinted from ref. 57 with permission, copyright 2016, American Chemical Society Publications). B) Single-cell sequencing by CTC-iChip. (a) A CTC sorting chip based on lateral flow offset, inertial focusing and magnetic field separation (reprinted from ref. 59 with permission, copyright 2013, license CC BY 4.0). (b) The experimental process of single-cell sequencing on the CTC-iChip (reprinted from ref. 61 with permission, copyright 2014, Elsevier B.V.). C) Operation of the microfluidic device. (a) Microscope image of array unit. (b) Microscope image of two trapped cells in the capture chambers (reprinted from ref. 62 with permission, copyright 2011, National Academy of Sciences). D) Design and operation of the microfluidic device. (a) Complete microfluidic device. The device contained 200 identical modules allow parallel analysis of 200 single cells; (b) workflow for single-cell digital PCR analysis of mRNA. Cells were chemical or heat lysed after being captured, and the released mRNA or miRNA were reverse transcribed to cDNA. PCR reagents were then injected into the device to mix with cDNA, and at last flew into the digital PCR array for detection (reprinted from ref. 63 with permission, copyright 2013, American Chemical Society Publications).

(Fig. 2A). When the encapsulated single cells were lysed and digested, the lysate-containing droplets would be merged with large water drops by the electric field to achieve dilution of the cell lysate, and then the collected droplets would undergo thermocycling to accomplish RT-PCR. By this method, specific cells could be identified from a mixed human cell population through the detection of multiple target genes. PCR also can occur in sol–gel switching agarose droplets to detect intracellular RNA.[66,67] In the research of Huifa Zhang et al.,[66] target RNA was detected by a reverse prime grafted to a linear polyacrylamide (LPA) chain (Fig. 2B). After the single-cell RT-PCR in droplets, the agarose droplets were solidified by reducing temperature below the gelling point. As the result of the copolymerization between acrylamide and LPA,

amplicons couldn't flow out of the solidified agarose, while the small molecule dye was permitted to enter the droplets and label the targets. The negative effect of cell lysate in agarose droplets could be inhibited by removing the cell lysate from droplets after sol–gel switching. In the research of Richard Novak et al.,[67] single cells were encapsulated together with lysate and primer-functionalized beads in agarose microdroplets. After cell lysis, target nucleic acids were connected with primer-functionalized beads by complementary base pairing (Fig. 2C). In the gel droplets under low temperature, the lysate was allowed to flow out, while the beads could be reserved in droplets because of its large size. At last, PCR amplification occurred and the chromosomal translocation t(14;18) in single cells were analyzed.
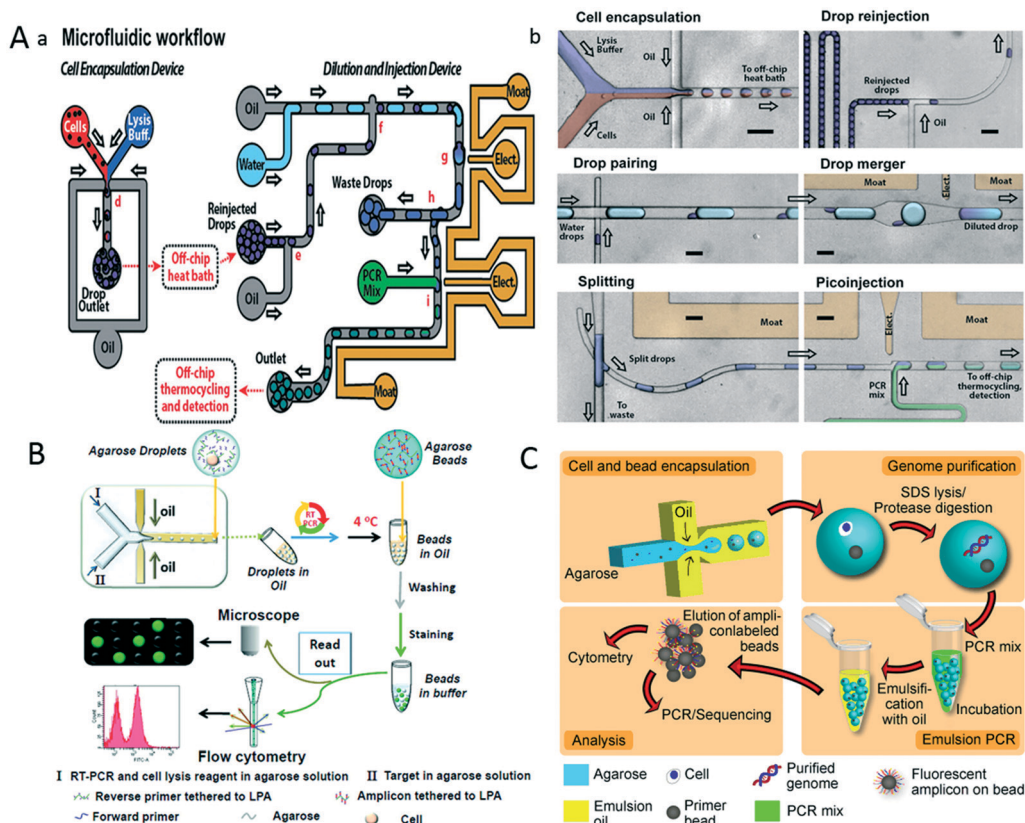
Fig. 2 A) Design and operation of the microfluidic chip for PCR. (a) Workflow of the microfluidic device. (b) Microscope images of the microfluidic device. (i) Droplets generated by microfluidic chip were used to encapsulate cells and lysis buffer. Then the droplets were heated and incubated out of the chip; (ii) droplets were reinjected into another chip and spaced with oil, then paired with dilution droplets and (iii) merged with electric field; (iv) a portion of the droplet was spit off and (v) merged with PCR mixture by a second electric field. After that, droplets were collected in a tube, for thermal cycling and analysis (reprinted from ref. 65 with permission, copyright 2013, American Chemical Society). B) Principle of intracellular RNA detection based on agarose droplets (reprinted from ref. 66 with permission, copyright 2012, American Chemical Society). C) The schematic diagram of agarose droplets encapsulating single cells and PCR reagents for the detection of intracellular nucleic acid (reprinted from ref. 67 with permission, copyright 2011, John Wiley and Sons).

To simultaneously measure expression levels of different kinds of nucleic acid molecules in single cells, a series encode techniques have been developed. For example, Saiful Islam et al.[68] designed a 5 bp random sequence tagged primer for capturing target RNA from lysed single mouse embryonic stem cells. (Fig. 3A) After PCR, a large number of amplicons were generated. In particular, a 5 bp random sequence serving as unique molecular identifiers (UMI) were able to distinguish up to 1024 mRNA because of the different arrangements of bases. Amplicons carrying the same UMI sequences were derived from the same allele. Thus, the specific recognition of single-cells mRNA could be realized by distinct UMIs. Evan Z. Macosko et al.[69] also detected intracellular mRNA by the combination of microfluidic technology and cell barcodes. First, mRNAs from the lysed cells could be bound to barcoded primer linked magnetic beads in droplets (Fig. 3B). Subsequently, the droplets were broken, and the beads were collected to complete reverse-transcribe and PCR amplification. At last, the generated amplicons were sequenced, and the number of mRNA transcripts were counted digitally in each cell. This method was applied to complex

mouse retina and retrieved 39 distinct populations which were closely related to cell types. The team subsequently further studied mouse retinal bipolar neurons by this technology, and fifteen types of bipolar cells were identified from approximately 25 000 single-cell transcriptomes.[70] Rapolas Zilionis et al.[71] utilizing similar a encoding technique combined with microfluidic chips, developed a single-cell mRNA detection method (Fig. 3C). Unlike the above method, barcode magnetic beads were replaced by barcode hydrogel beads, which were produced by covalent incorporation with acrydite-modified DNA primer and hydrogel mesh. Then the barcoding primers would be released from the hydrogel beads by photocleavage, followed by the initiate of mRNA reverse transcription. This barcode was also composed of cell barcode and unique molecular identifier (UMI), which were devoted to the distinction of different mRNA and the source cells.

2.1.2 Other amplification methods for single-cell nucleic acids analysis. In nucleic acids detection, PCR requires raising and lowering the temperature repeatedly to separate DNA strands which is complicated and challenging for single-cell
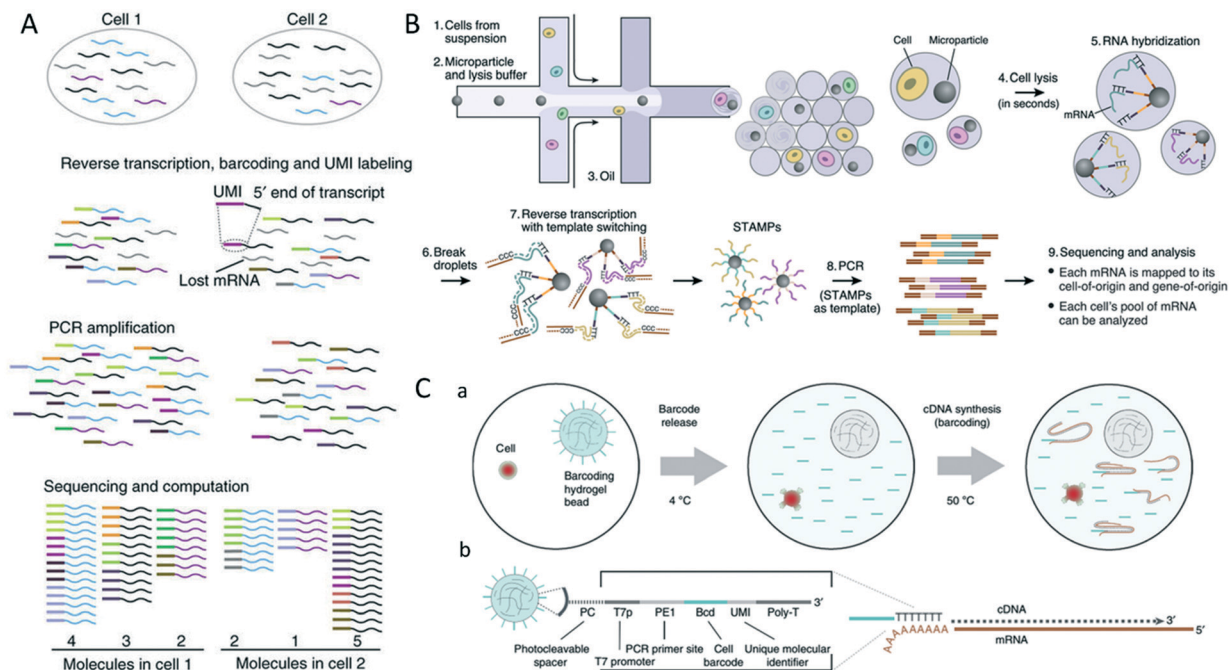
Fig. 3 A) The schematic of mRNA detection combined with UMI. Different colors represent different mRNA in cells. UMI stands for unique molecular identifier (reprinted from ref. 68 with permission, copyright 2013, Nature Publishing Group). B) The schematic of mRNA sequencing in individual cells. After the cells, encoding beads and cell lysate were encapsulated into droplets, the cells would be lysed to release mRNA, which could be bonded to the coding primer on the bead surface. Then the droplets were broken, collecting mRNA-bound microparticles to perform reverse transcription. Finally, the amplified cDNA could be sequenced by a sequencer. (a) Reverse transcription in droplets. After the cells and hydrogel beads were encapsulated in droplets, the coding primers were released from the beads by irradiation with a >350 nm UV light source. Then, the captured mRNA underwent a reverse transcription reaction. (b) Coded hydrogel. Brown line represents mRNA and gray line indicates cDNA (reprinted from ref. 69 with permission, copyright 2015, Elsevier). C) The process of single-cell transcription and barcoding in droplets (reprinted from ref. 71 with permission, copyright 2016, Nature Publishing Group).

analysis.[72] To avoid the thermocycling, several isothermal amplification techniques have been developed. Rolling-circle replication originally was discovered in single-stranded DNA (ssDNA) bacteriophages of *E. coli*, by which a long, repetitive ss-product was synthesized isothermally utilizing a circular template and polymerase.[73,74] Based on such a principle, rolling circle amplification (RCA) as a signal amplification technique was developed. In RCA, circular DNA is used as a duplicate template for primer extension with the catalysis of DNA polymerase. Then, a large number of linear single stranded DNA molecules with repetitive and complementary sequence to circular template can be generated.[75] So far, RCA has been widely applied for signal amplification in gene analysis.[46,75–78] Because of the thermostatic reacted conditions of RCA, it is well adapted to be performed in microfluidic chips. For example, Linas Mazutis *et al.*[53] have measured *lacZ* gene in single cells by RCA with a microfluidic system (Fig. 4A). First, a large number of droplets packaging single cells, lysate, RCA mix, and intercalating fluorochrome were generated by a microfluidic system. Then the droplets were collected to complete off-chip RCA reaction and reinjected into an analysis chip to monitor the fluorescence. The authors also detected the activity of β-galactosidase by an *in vitro* transcription/translation (IVT) reaction. Meiye Wu *et al.*[79] utilized rolling-circle amplification for miR 155 detec-

tion in single Jarkat cells with a 10-chamber microfluidic chip combined with flow cytometry (Fig. 4B). The Jarkat cells were captured through Cell Tak™ pre-coated on the surface of a microchannel. After cell permeabilization, intracellular miR 155 was identified by a complementary probe and forming a circle DNA template by the complementary hybridization of oligonucleotides. Subsequent rolling circle amplification was performed by the circular template and Phi29 bacterial polymerase. By adding FITC-labeled oligonucleotide detection probes complementary to the amplified production, the microRNA could be detected by a green fluorescence measurement. Multiple displacement amplification (MDA) is also a thermostatic total genome amplification method proposed in recent years.[80] After combining random hexamers primer and template DNA by annealing, strand-displacement synthesis is catalyzed by Phi 29 DNA polymerase or Bst DNA polymerase at a constant temperature, thus generating high molecular weight DNA strands. Then, the strands are displaced and the primers are bound to the newly synthesized DNA to participate in the next polymerization.[81,82] This method can produce uniform whole genome sequences and minimal amplification errors, which ensure the information consistency between templates and the productions. Because of its unique advantages, this method has become a popular whole genome sequence method for single-cell nucleic acids
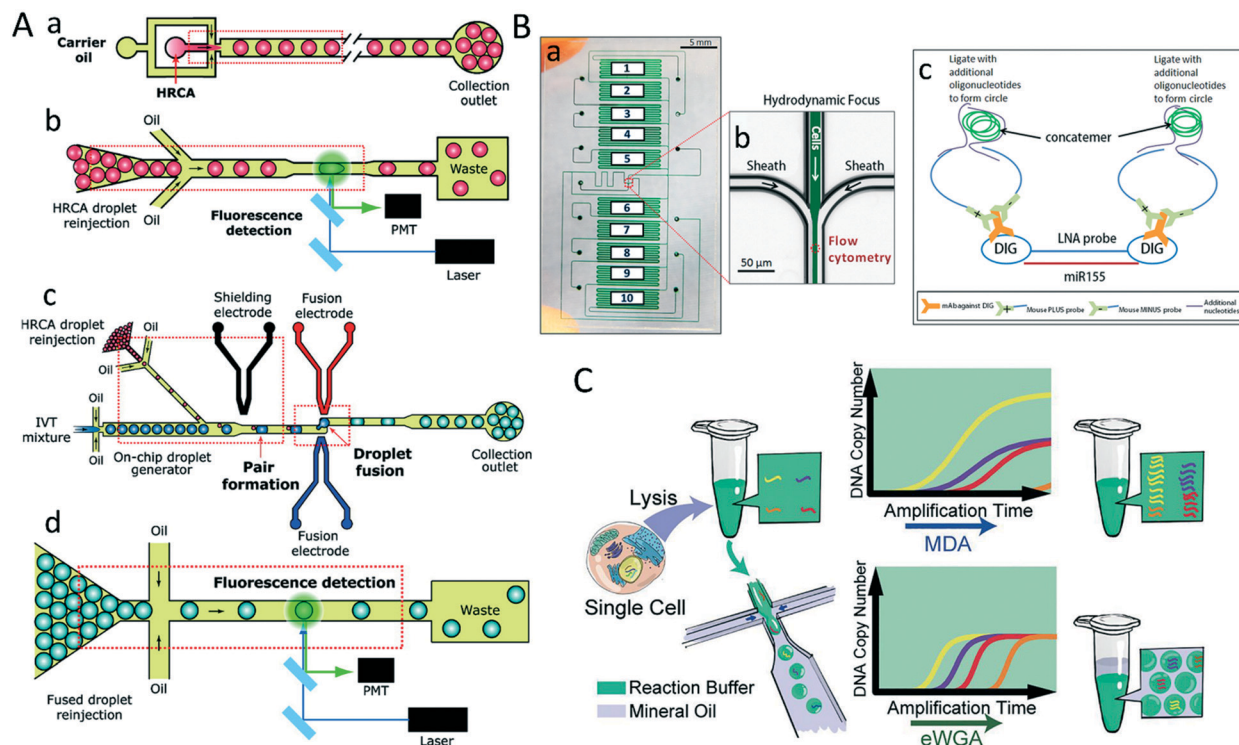
Fig. 4 A) The microfluidic device. (a) HRCA droplet generation device. (b) HRCA droplet analysis device. After off-chip incubation, HRCA droplets were reinjected into the device and separated with fluorinated oil. The fluorescence was monitored using PMT. (c) Device for droplets fusion. The reloaded HRCA droplets were paired with IVT droplets and fused by an AC electric field (30 kHz, 600 V). (d) Fused drop analysis device (reprinted from ref. 53 with permission, copyright 2009, American Chemical Society). B) Microfluidic chip and LNA flow-FISH method schematic. (a) A diagram of microfluidic chip with a 10 micro-chamber. (b) The process of hydrodynamic focus. (c) The principle of signal amplification. First, miRNA 155 was hybridized to the LNA probe. The DIG labelled on both ends of the LNA probe were recognized by the anti-DIG mAb while the two RCA probe (+ and −) could be combined to the anti-DIG mAb respectively, and an additional oligonucleotide sequence were ligated to form a template for rolling circle amplification (reprinted from ref. 79 with permission, copyright 2013, license CC BY 4.0). C) The miRNA detection schematic based on dd-PCR (reprinted from ref. 83 with permission, copyright 2015, National Academy of Sciences).

analysis. For example, Yusi Fu *et al.*[83] developed a MDA method that combines with microfluidic droplets to achieve measurement of mRNA in single cells (Fig. 4C). They distributed single-cell genomic DNA fragments into a large number of droplets, a few of which contained one DNA fragment and MDA reagents. For amplification of the whole emulsion system, the MDA reaction was conducted in each droplet containing the fragment. Although there were significant differences in dynamics between these independent droplets, it would reach saturation of amplification and the differences in amplification would be significantly minimized. Thus, compared to conventional MDA, uniformity and accuracy of the MDA could be improved.

2.2 Fluorescence labelling and sensing of proteins

Intracellular protein molecules such as various receptor proteins, signal enzyme molecules, and structural proteins *etc.* play critical roles in maintaining the stabilization of cell structure, transducing important signals, and regulating cell functions.[84,85] There are approximately 100 000 different kinds of proteins in cells, and their contents are extremely varied. The expressions of some structural proteins are up to

about 10^8 molecules, while some receptor proteins may be less than 100 molecules.[21] Besides, specific biological functions of proteins are intrinsically encoded in their complex three-dimensional structures.[86] However, unlike nucleic acids which are specifically recognized by complementary sequences and amplified through various DNA amplification reactions, it is challenging to design specific probes and signal amplification methods for single-cell analysis of proteins.[84] Currently, protein recognition and fluorescence labelling are mainly through linking a fluorescent protein to target proteins in living cells by a gene transfection technique,[87] and binding target proteins with specificity labeled-antibodies by an immunofluorescence method.[88–90] What's more, fluorescence labelling methods based on aptamer and small molecular fluorescence probes have also been promoted.[30,91] Among these methods, fluorescent protein labelling has strong specificity. Combined with a fluorescence imaging technique, it can be used for studying expression dynamics and the distribution of proteins in living cells. In microfluidic single-cells analysis, this kind of labelling method is rarely reported because of lesser needs of single-cell manipulation by a microfluidic chip. Immunofluorescence methods can achieve identification and labelling of target proteins by

the combination of antigens and fluorescence tagged antibodies. Moreover, protein sensing and fluorescent signal amplification can also be realized by attaching an enzyme to antibodies which are able to catalyse a large number of substrate molecules producing fluorescence. This is the main method for single-cell proteins analysis on microfluidic chips. As to the sensing and labelling method based on an aptamer, "off–on" switch of a fluorescent signal and signal amplification can be realized by the probe design and various DNA amplification methods on the basis of DNA characteristics of aptamers. This usually requires a confined space in the microfluidic chips to isolate and enrich single cell signals. Compared to antibodies and aptamers for the identification of proteins, the design and synthesis of organic small molecule fluorescent probes are relatively complicated. Aiming at different structures and functions of proteins, different design strategies of specific target groups and reporter groups are demanded.

2.2.1 Immunofluorescence for single-cell proteins analysis.

Immunofluorescence is one of the most commonly used methods for protein detection. Its fundamental principle is complementarity determining regions of antibody accounting for the basic specific combination with a particular antigenic determinant structure. After fluorophores are chemically linked to antibodies, qualitative and quantitative information of target proteins can be obtained by the immunoreaction between antibodies and antigens.[92] Homogeneous immunoassay and heterogeneous immunoassay are both used in the single-cells proteins analysis on microfluidic chips. After antigen–antibody interactions, antigen–antibody complexes and residual free antibodies both exist in a reaction system. Usually, they should be separated by two phases and then measured in a heterogeneous immunoassay. In the other case, separation is not required on account of different properties of antigen–antibody complexes and free antibodies. Hence, measurements can be conducted directly in the solution which is regarded as a homogeneous immunoassay.[88,93] For instance, based on high efficiency microfluidic chip electrophoresis technology, antigen–antibody complexes and residual free antibodies with different charge-to-mass ratios can be distinguished by migration time. This is typical with a homogeneous immunoassay for single-cell. Based on the electrophoretic separation technique, Huang et al.[13] quantified β2 adrenergic receptors in insect cells (SF9) (Fig. 5A). Through the design of a three-state valve on microchips, single cell capture and lysis on a microchip could be achieved successively. Then the released proteins were labelled with fluorescent tagged antibodies, and protein-antibody complexes and free antibodies were separated according to different migration times by chip electrophoresis. At the end of the separation channel, the detection of low-copy number proteins were realized with a single-molecule fluorescence counting device.

Heterogeneous immunoassay is the main method used in microfluidic single-cell protein analysis. In this process, target proteins are captured by antibody-modified microchip substrates or antibody linked beads. Besides, cells with target proteins can also be regarded as solid phase substrates for an immunoreaction. In single-cell analysis, beads or glass substrate of commonly used microchips can be chemically modified conveniently by alkylation, amidation, epoxidation, etc. to bind antibodies to a specific location on the chip substrate.[94–98] Thus, target proteins in single isolated cells, or secreted by cells, can be caught on the chip and then labelled and detected by a fluorescent second antibody. In this process, the separation efficiency of free antibodies can be greatly improved by multiple-step washing on a microfluidic chip and reagent consumption can be effectively reduced.[99,100]

For the assay of intracellular proteins, cells should be lysed to release inner proteins followed by fluorescent labelling by immunoreaction and detected. Yasuhiro Sasuga et al.[101] designed a simple array chip of microwells to detected target proteins hemagglutinin (HA) and glutathione S-transferase (GST) by antibody-conjugated microbeads (Fig. 5B). Single cells were trapped in microwells and subsequently lysed. Then, target proteins could be captured by antibody-conjugated microbeads and recognized by adding Cy3-labeled second antibodies. Heterogeneous immunoassay was also used by Ali Salehi-Reyhani et al.[30] for measuring the amount of human tumour suppressor protein p53 in the breast cancer cell line MDA-MB-468 (Fig. 5C). Single cells were first captured in the microchamber and then lysed by a shock wave caused by laser-induced micro-cavitation, then the target proteins were grasped by antibodies pre-modified on the bottom of the chip. Next, fluorophore-labelled antibodies were introduced to form a sandwich structure and to detect the fluorescent signal by total internal reflection microscopy. In order to improve detection sensitivity, K. Eyer et al.[100] incorporated enzyme-linked immunosorbent assays (ELISA) with microfluidic chips for detecting content of enzyme glycerylaldehyde 3-phosphate dehydrogenase (GAPDH) in single U937 cells and HEK 293 cells (Fig. 5D). They captured and isolated single cells through ring-shaped valve controlled microchambers. After lysis, GAPDH released and immobilized by the antibodies modified on the bottom of chip, and HRP-labeled anti-GAPDH combined with GAPDH formed a sandwich structure. Then, HRP catalyzed the substrate to converse to a fluorescent product. Target proteins were able to be detected by TIRF microscopy. In this method, multiple reagent injection and washing steps were required for ELISA; the design of a ring valve controlled chip can achieve precise fluid manipulation and satisfy that requirement of ELISA.

Except for intracellular proteins, secreted proteins, such as salivary amylase, pepsase, some hormones etc., playing roles by secreting out of the cells, are another kind of potential rich source of biomarkers.[102–105] Single-cell analysis of secreted proteins is helpful for better understanding of the functional mechanism of signaling pathways in organisms.[102,106–108] Dong-Sik Shin et al.[109] analysed extracellular secreted proteins TNF-a in single CD4 and CD8 T cells
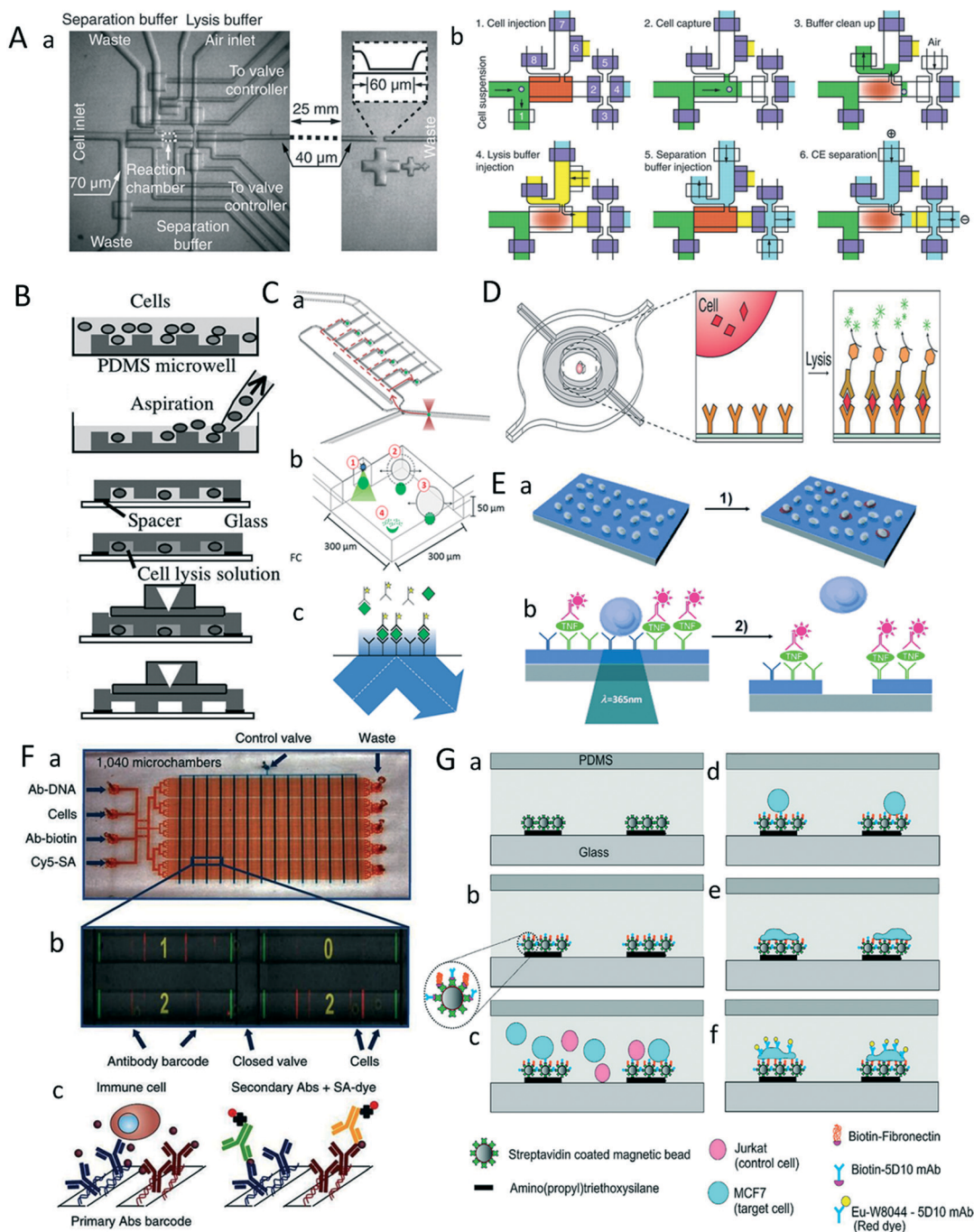
Fig. 5 A) The single-cell analysis chip. (a) The single-cell manipulation section on microfluidic chip. (b) Analysis procedure for a mammalian or in-sect cell (reprinted from ref. 13 with permission, copyright 2007, The American Association for the Advancement of Science). B) Single cell capture and lysis process (reprinted from ref. 101 with permission, copyright 2008, American Chemical Society). C) The schematic of single cell capture (a), lysis (b) and intracellular protein detection (c) (reprinted from ref. 30 with permission, copyright 2011, Royal Society of Chemistry). D) The sche-matic of enzyme-linked immunosorbent assays for detection of proteins in single cells (reprinted from ref. 100 with permission, copyright 2013, American Chemical Society). E) The schematic of single-cell capture and secreted proteins detection. (a) Photodegradable hydrogel was modifica-tion on the glass substrate. Then, antibodies for single cells and proteins capture were printed. (b) The process of single-cell release (reprinted from ref. 109 with permission, copyright 2014, John Wiley and Sons). F) The schematic of single cell barcode chip (SCBC) for the analysis of sec-reted proteins. (a) Microscope image of SCBC. (b) Microscope image of microchamber. Isolated cells were recognized with fluorophore-labeled antibody barcodes. (c) Detection principle of cell secreted proteins (reprinted from ref. 89 with permission, copyright 2011, Nature Publishing Group). G) The schematic of specific capture of MCF-7 cells by self-assembled magnetic beads in microfluidic channels. (a) Modified self-assembly beads on the channel. (b) Biotinylated antibodies and biotinylated fibronectin were integrated onto the bead surface. (c) The cell suspension containing MCF-7 and Jurkat cells was incubated with magnetic beads. (d) After washing, only MCF-7 cells were specifically captured by magnetic beads. (e) MCF-7 cells adhered and grew on magnetic beads. (f) The fluorescent dye-labeled 5D10 antibody on the surface of MCF-7 was detected (reprinted from ref. 113 with permission, copyright 2010, American Chemical Society).

through an antibody-modified microchip substrate (Fig. 5E). First, they grafted the photogel on acrylated glass surfaces, then printed two kinds of antibodies, respectively, for capturing cells and secreted proteins. CD4 and CD8 T cells could be immobilized on the microchip by antigen–antibody interactions. Once proteins TNF-α were secreted, they would be caught by modified antibodies. Introducing fluorophore-labelled anti-TNF-a, measurement of TNF-α could be achieved with a fluorescence microscope. Simultaneous detection of multiple types of proteins in single cells is conducive to better understanding of the correlation among various proteins and their synergistic effects in cells, and could provide both fundamental immunobiological information and clinically relevant data.[89] To realize simultaneous detection of multiple secreted proteins in one cell, James R Heath et al.[89] designed a single-cell barcode chip (SCBC) for the assessment of 12 kinds of secreted proteins at the single-cell level (Fig. 5F). The chip was composed of 1040 3 nL volume microchambers in which single cells were loaded. Antibodies were modified on the bottoms of microchambers, and each kind of antibody formed a column, which constituted the parallel stripy barcode array. Different proteins secreted by individual cells could be captured by the antibody barcode and next labelled by other fluorophore-linked antibodies forming sandwich structures. In this method, the types of secreted proteins could be determined according to the location of the fluorescent strips. Meanwhile, the amount of proteins could also be measured according to the intensity of the fluorescence. Similarly, Yu Zhang et al.[110] detected 8 proteins in three lung cancer cell lines (A549, H1650, and H975) by a barcode microchip. They fabricated an antibody microchip of barcode arrays, which then covered the nanowell chip to form an enclosed reaction chamber. Thus, target proteins would bind to barcode antibodies on the ceilings of chambers.

Membrane proteins are another kind of important protein in cell expression. Some of them adhere to the membrane surface by the inter-attraction between charged amino acids or groups in peptides and polar lipid molecules in a cell membrane, while others can be embedded in the membrane by a hydrophobic α helical structure.[90] Different membrane proteins undertake diverse physiological functions in living cells. For example, membrane transport proteins directly participate in molecular transport; receptor proteins are used to transduce signals by binding to ligands; tubulin is closely related to cell movement and so on. Moreover, the changes of membrane proteins are closely related to many diseases.[111] They are also major targets of cancer drugs as potential cancer markers.[112] Hence, single-cell analysis of membrane proteins has attracted extensive attention. For example, Venkataragavalu Sivagnanam et al.[113] have detected membrane proteins 5D10 in single MCF-7 cells using a heterogeneous immunofluorescence assay on a modified microchip. The cells which expressed target antigens were captured by 5D10 antibody linked on the microchannel. Subsequently, proteins 5D10 were marked by fluorophore-labeled 5D10 antibodies. Finally, a fluorescent microscope was utilized for imaging detection (Fig. 5G).

Apart from the antibody-modified microchip substrates utilized for proteins capture, the antibody-modified magnetic beads and cells themselves are the other two substrates in a heterogeneous immunoassay. They are usually combined with a microdroplets technique to perform single-cell proteins analysis. By precisely controlling the dimension of the microfluidic chip channels and the flow rate of multiphasic flows such as oil phase and water phase, size-controlled and stabilized monodisperse microdroplets can be generated with high throughput.[114–118] In the process of droplets forming, not only single-cell capture and isolation can be achieved, but also various reagents which are required for single-cell analysis can be simultaneously encapsulated in droplets. In addition, the small volume of the confined space of droplets can function as an independent microreactor for the immunoreaction to accelerate the reaction rate and enrich the signal. For instance, V. Chokkalingam et al.[119] used functionalized antibodies-modified beads incorporated with droplet-based microfluidic approach for analysis of multiple cytokine secretion of single T-cells (Fig. 6A). First, L-2, IFN-γ, and TNF-α secreted by single T-cells can be bound to functionalized capture beads in individual agarose droplets. Then, under low temperature, agarose droplets gelled forming stable and solid gel beads, which permitted entrance of multicolour fluorophore-labeled antibodies. Whereupon, simultaneous detection of three proteins were realized. In this method, in order to separate antibody-bind protein complex and free antibody, an agarose material solidified under low temperature is required and repeated washing of free fluorophore-labelled antibodies are necessary. Linas Mazutis et al.[120] established a single-cell secreted protein detection method combining capture beads and droplet-based microfluidic systems, which require no washing step (Fig. 6B). In a droplet, secreted proteins of a single cell were firstly captured on the surface of antibody-linked beads, subsequently fluorophore-labeled antibodies were bound to the secreted proteins. By the sandwich reaction, the fluorophore-labeled antibodies assembled on the bead surface, and a stronger distinguishable signal was generated compared to the background. Then, the target detection would be achieved.

Using the cells in droplets as the solid phase of the immunoreaction is mainly adapted for the detection of membrane proteins. This is because cell lysis is non-essential in cell membrane protein detection; the generated protein-antibody complex is directly attached to the cell surface. For example, Tania Konry et al.[121] using PC3 cells as solid phase, detected proteins EpCAM on cell surfaces. First, PC3 cells, biotinylated-antibodies, biotinylated DNA primer, and RCA reagents were encapsulated in a microfluidic nanoliter reaction droplet (Fig. 6C). Membrane proteins combined with specific biotinylated-antibodies that later bound to DNA primer via biotin-avidin bridge; subsequently, RCA was triggered and a long one-dimensional DNA substrate with hundreds to millions of bases were produced. With information from cell membrane proteins, EpCAM could be reflected by fluorophore-labeled nucleotides synthesized on long strand
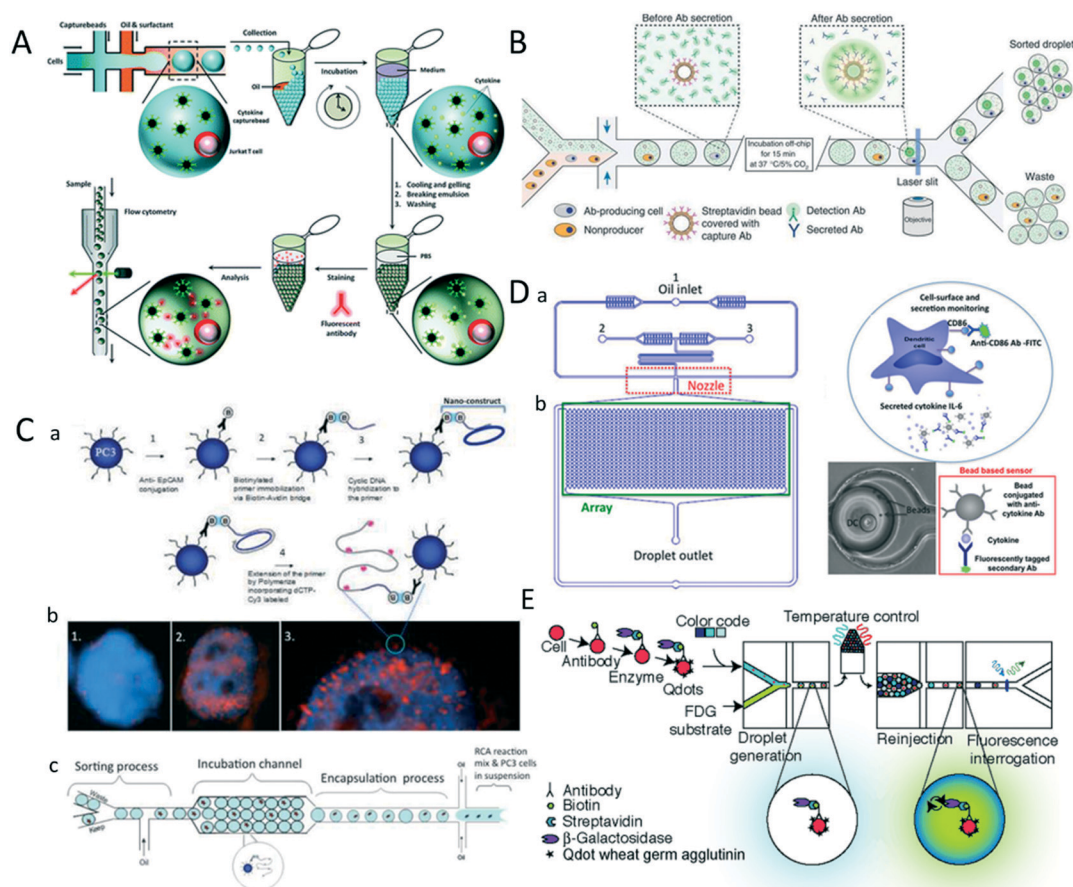
Fig. 6 A) Workflow of microfluidic method for single-cell encapsulation and detection of secreted cytokines (reprinted from ref. 119 with permission, copyright 2013, Royal Society of Chemistry). B) The principle of secreted proteins detection by antibody modified magnetic beads in droplets. Two types of cells (orange and gray) and antibodies (capture antibody modified on magnetic beads and detection antibody labeled with fluorophore) were introduced from both entrances of the chip. After collecting droplets and incubation for 15 minutes, droplets were reinjected into a second chip, the cells were sorted by fluorescence-activated droplet sorting (reprinted from ref. 120 with permission, copyright 2013, Nature Publishing Group). C) Schematic of the detection of target membrane proteins. (a) Diagram of rolling circle amplification; (b) microscope images of a PC3 cell immobilized on a glass slide and stained with DAPI; (c) the microfluidic chips for droplets generation (reprinted from ref. 121 with permission, copyright 2013, John Wiley and Sons). D) The work principle of simultaneous detection of membrane and secreted proteins on the microfluidic chip. (a) Diagram of the microfluidic chip. (b) Schematic of microfluidic reactions for simultaneous monitoring membrane and secreted proteins. The bright field is a single cell and beads co-encapsulated in droplet (reprinted from ref. 122 with permission, copyright 2013, Nature Publishing Group). E) Schematic of experimental measurement based on ELISA. Antibody-coupled enzymes are used to label specific proteins on the cell surface. Subsequently, cells are coded and introduced into the chip with fluorescent enzyme substrate to form droplets. After incubation for a period of time, fluorescence in droplets was analysed (reprinted from ref. 123 with permission, copyright 2013, John Wiley and Sons).

DNA. This method could realize the specific recognition of a target protein by immunoreaction, and the design of rolling circle amplification could greatly improve the sensitivity of the test, which is especially suitable for analysing low-abundance membrane proteins in single cells. T. Konry et al.[122] made use of magnetic beads and cells in the droplet as a solid phase substrate for the simultaneous immunoassay of membrane protein and cell secreted protein (Fig. 6D). During the detecting process, single dendritic cells were isolated in micro-droplets, secreted IL-6 was detected by the conjugation of captured Abs (anti-IL-6 Abs) on the bead surface and fluorophore-labeled detection Abs (anti-IL-6-FITC Abs). Meanwhile, membrane proteins were detected by CD86 fluorophore-labeled detection antibodies (anti-CD86-FITC Abs) localized on the cell surface.

Besides, enzyme-linked immunosorbent assay (ELISA) also can be used in microfluidic heterogeneous immunoassay because of its high sensitivity. Haakan N. Joensson et al.[123] developed a high-sensitive single-cell membrane protein detection method based on ELISA (Fig. 6E). First, cell membrane proteins were specifically labelled by antibodies, and then streptavidin-coupled β-galactosidase was bound to the biotinylated antibodies. Next, single enzyme-labeled cells and fluorogenic substrate FDG were co-encapsulated in monodisperse aqueous droplets produced by microchips. The enzymic catalytic reaction between enzymes and the FDG substrates could amplify the signal of membrane proteins in single cells, leading to high detection sensitivity.

2.2.2 Aptamer and small-molecule fluorescent probes for single-cell proteins analysis. Aptamers are single stranded

RNAs or DNAs with particular three-dimensional structures, which have strong capacity for specifically binding and recognizing the target with high equilibrium dissociation constant (K_d) of the aptamers–target complex, commonly in the nanomolar-to-picomolar range.[124,125] Since its function is similar to antibodies, aptamers are also known as chemical antibodies.[126] Aptamers are flexible in the applications because of their good thermal stability, synthetic accessibility, high specificity, and affinity for interacting with target molecules.[127,128] In the analysis of cell proteins, aptamers were often used for targeting cell membrane proteins to achieve imaging and quantified detection.[128,129] On a microfluidic chip, aptamers were usually regarded as specifically captured molecules to realize single-cell capture and sorting by specifically binding to membrane proteins. Especially, they played important roles in the separation and identification of circulating tumor cells (CTCs).[130,131] For single-cell component analysis, identification and labelling of target proteins by aptamers were less frequently reported. Our group[15] has identified membrane proteins PTK7 by taking advantage of aptamers sgc8, and analyzing the expression of PTK7 in single HeLa cells based on the micro-droplet technique (Fig. 7A). In order to enhance detection sensitivity, a nicking enzyme assisting signal amplification was applied. In this work, we not only used aptamer sgc8 as an identifier of membrane proteins, but also added a DNA segment onto the sequence of sgc8 to make it take part in the subsequent amplified reaction. Except for this research, other aptamers have also been extended and modified to participate in various DNA amplification reactions, like RCA and PCR to greatly enhance the detection sensitivity.[132,133] The extensibility and flexibility provide aptamers more potential in single-cell protein analysis.

Small molecule fluorescence probe refers to a kind of small molecular substance that can interact with the target molecule specifically, resulting in changes of its fluorescence properties.[134–136] Compared to the acquisition of antibodies and aptamers for protein labels, the design and synthesis of small molecular fluorescence probes for fluorescence detection of cell proteins are relatively complicated. Different target groups and fluorescence signal reporting groups are needed to be designed for different characteristics of proteins. So far, many small molecular fluorescence probes have been synthesized for cell protein detection.[137,138] As these probes are not universal, in terms of a large amount of protein species, the number of proteins that can be labeled and detected by such probes is still relatively less, especially for their applications in microfluidic chips at a single-cell level. In this field, Fei Xu et al.[138] synthesized a fluorescent ABP probe which was capable of targeting GB1 receptors. This ABP probe consists of three parts: a "warhead" was used to recognize the active domain of the GB1 subunit, which was similar to GB1 receptor antagonists; a fluorescent BODIPY which was responsible for subsequent fluorescence measurements, and a linker between the fluorescent tag and "warhead" (Fig. 7B). A single neuron extracted from a mouse was labeled by ABP probes and located in a PDMS chip. Because the BODIPY was a signal reporting group that was "always-on", capillary electrophoresis-laser induced fluorescence (CE-LIF) detection was required to separate the protein-probe complexes and free probes. Thus, the GB1 receptor could be detected. In addition to "always-on" small molecule fluorescence probes, "off–on" probes are more popular because there is no need to separate the products and probes in the labelling process. Typical representatives of these small molecule probes is a substrate for enzyme detection; this is a kind of molecule with weak or no fluorescence originally, but a strong fluorescent signal can be generated because of changes in its molecular structure after being catalyzed by proteases.[91,100] Furthermore, signal amplification can be achieved by the production of a large number of fluorescent molecules catalyzed by one protein.[100,139] By this principle, the enzyme molecules inside a single cell can be detected with high sensitivity. For example, Jung-uk Shim et al.[91] quantified the content of alkaline phosphatase (AP) by fluorescent products formed during the enzymatic catalysis in single E. coli cells in microdroplets. Linas Mazutis et al.[53] also realized the detection of β-galactosidase by catalysing its specific non-fluorescent deoxyglucose substrates.

2.2.3 Gene transfection for single-cell proteins analysis. Gene transfection technology means delivering purified DNA containing a target gene into cells; subsequently the target
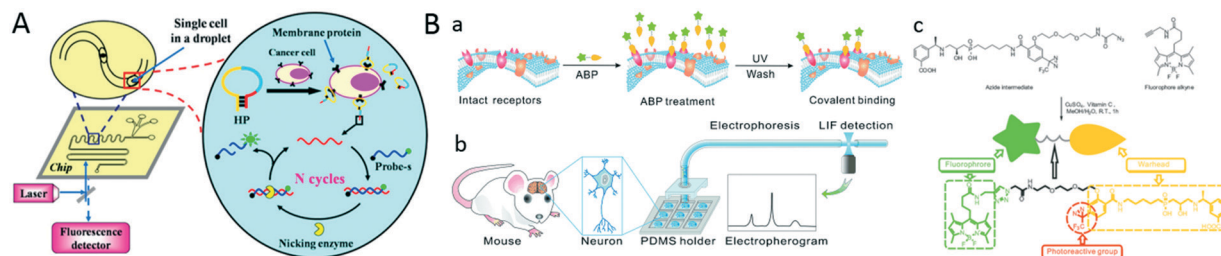

Fig. 7 A) The process that single-cells encapsulated in droplet on a microfluidic chip and the principle of nicking enzyme-assisted fluorescence signal amplification for target membrane proteins (reprinted from ref. 15 with permission, copyright 2014, American Chemical Society). B) The schematic of single-cell chemical proteomics (SCCP) detection strategy. (a) The labelling process of ABP; (b) single neuron extracted from small mice was injected into capillaries for CE-LIF analysis to study specific receptors which could be functionally labeled by an ABP probe. (c) Synthesis of ABP probe (reprinted from ref. 138 with permission, copyright 2014, John Wiley and Sons).

gene can express in these cells. Benefitting from high biocompatibility and abundant species of fluorescence proteins currently, a large number of studies have been conducted for labelling and detecting target proteins using gene transfection techniques by which fluorescent proteins can be linked to target proteins.[140,141] The detailed process involves connecting the fluorescent protein gene to the target genes; after transcription, fluorescent proteins can be fused with the target proteins.[142,143] Like attaching a fluorescent tag to the target protein, it enables the research of temporal and spatial variations of protein expression in living cells. Various fluorescence imaging techniques are the main detection means for these labelled proteins. By combining with a microfluidic chip, many imaging analytical methods of proteins at a single-cell level have been developed. A. Huebner et al.[87] have simply detected the expression of yellow fluorescent proteins in E. coli cells trapped in microdroplets. Subsequently, Jung-uk Shim et al.[91] transfected E. coli cells to express fluorescent proteins mRFP1 and monitored the their expression level in individual E. coli cells on a microfluidic chip with an epi-fluorescence microscope. Through tagging the fluorescent proteins to target proteins, information of signaling pathways in cells can also be reflected. Min Cheol Park et al.[144] fused a green fluorescent protein to signal a specific promoter (Pfus1) for researching the mating MAPK signaling pathway. The transfected yeast cells were trapped by a microwell-based cell array and the green fluorescent proteins could be observed to research the mating MAPK signaling pathway.

2.3 Fluorescence labelling and sensing of small molecules

There are various active small molecules in cells, including metabolites, inorganic ions, organic small molecules, and a few gas molecules. Such molecules are widely involved in signaling pathways and play regulatory roles in physiologic functions. For example, Ca^{2+} is central to cardiac contraction and regulates hormone secretion and neurotransmitters release.[145] Intracellular reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$) and H_2O_2, etc., participate in immune system control, cell growth, and metabolism regulation, synthesis of important biological substances, and other regulation of physiological processes.[136,137] H^+ in cells maintains the homeostasis of intracellular pH and is related to the function of ATPase.[146] Hydrogen sulfide (H_2S), nitric oxide (NO), and carbon monoxide (CO) are regarded as the third recognized gasotransmitter, playing important parts in the cardiovascular, immune, and nervous systems.[147–150] The molecular weights of these active molecules are commonly relatively small (generally less than 100 MW). Different kinds of small molecules in cells possess an obvious variety in distribution and contents. At present, organic small-molecule fluorescent probes have emerged as a powerful analytical tool to mark and sense various small biological molecules.[151–163] These fluorescent probes usually consist of three parts: a receptor for reactive small molecules recognition, a fluorophore for signal generation, and a spacer for linking receptor and fluorophore. Among which, the receptor

determines the selectivity and specificity of probes and the fluorophore determines the luminescence characteristics. The chemical environment of a fluorophore will alter when a receptor is combined with an analyte, which contributes to the shift of the probe's fluorescence spectrum or increases/decreases fluorescence intensity.[164]

Currently, small molecules detection in single cells based on microfluidic chips mainly relies on labelling with organic fluorescent probes and combining with fluorescence microscope imaging or CE-LIF to acquired fluorescent signals. Because of the importance of Ca^{2+} in signal transduction, muscle contraction and other physiological process,[165–168] as well as the wide development of Ca^{2+} fluorescent probes,[169] a lot of work has been implemented for fluorescence imaging studies of Ca^{2+} in single cells. For example, Xiujun Li et al.[170] retained a single cardiomyocyte on a microchip with a V-shaped cell retention structure (MV1) and specifically imaged intracellular labelled Ca^{2+} with Fluo 4-AM probes (Fig. 8A(a)). To further study the drug response of single cardiomyocytes, the author improved the old microchip (MV1) by adding a short leading channel on the cell retention chamber, which was helpful for multiple-step liquid delivery (MV2) (Fig. 8A(b)).[171] Then, the dynamic $[Ca^{2+}]_i$ mobilization under different drug stimulation could be measured in single by Fluo 4-AM fluorescent probes. Kwanghun Chung and Loice Chingozha et al.[172,173] detected calcium oscillatory behaviour in single Jurkat T cells with fluorescent probes Fluo 3-AM on a high-efficiency single cell capture microchip (Fig. 8B). Michael Kirschbaum et al.[174] used fluorescent probe Fura-2/AM to detect immediate cytosolic Ca^{2+} response in activated and non-activated cells (Fig. 8C). In a lab-on-chip system, single Jurket T cells were combined with antibody-coated (anti-CD3, anti-CD28) microbeads by an antigen–antibody interaction and subsequently T cells would be activated by antibodies on the surface of beads, causing changes of intracellular Ca^{2+}. Then, Ca^{2+} was stained with Fura-2/AM, followed by observing the fluorescent signal. Next, the expression of the activation marker molecule CD69 would be observed off the chip by labelling anti-CD69-FITC. Thus, the simultaneous analysis of short-term Ca^{2+} responses and long-term protein CD69 expression could be realized. Apart from intracellular Ca^{2+}, other active small molecules can also be detected by microfluidic chips combined with a confocal microscope.[175,176] For example, Ning Gao et al.[176] designed a microchip with a simple U-shaped single-channel for fluid control to simultaneously quantitate glutathione (GSH) and reactive oxygen species (ROS) in individual cells by twice imaging of confocal microscopy.

Notably, only molecules with less overlapped fluorescence spectra can be distinguished by fluorescence imaging. Ideally, using distinguishable fluorescent probes and selecting appropriate fluorescent detection ranges can realize simultaneous detection of three or four kinds of components.[177,178] Once fluorescence emission spectra of more different label molecules are overlapped, it may be difficult to make intuitive and accurate analysis of them.[179] Chip electrophoresis combined with Laser induced fluorescence technology has
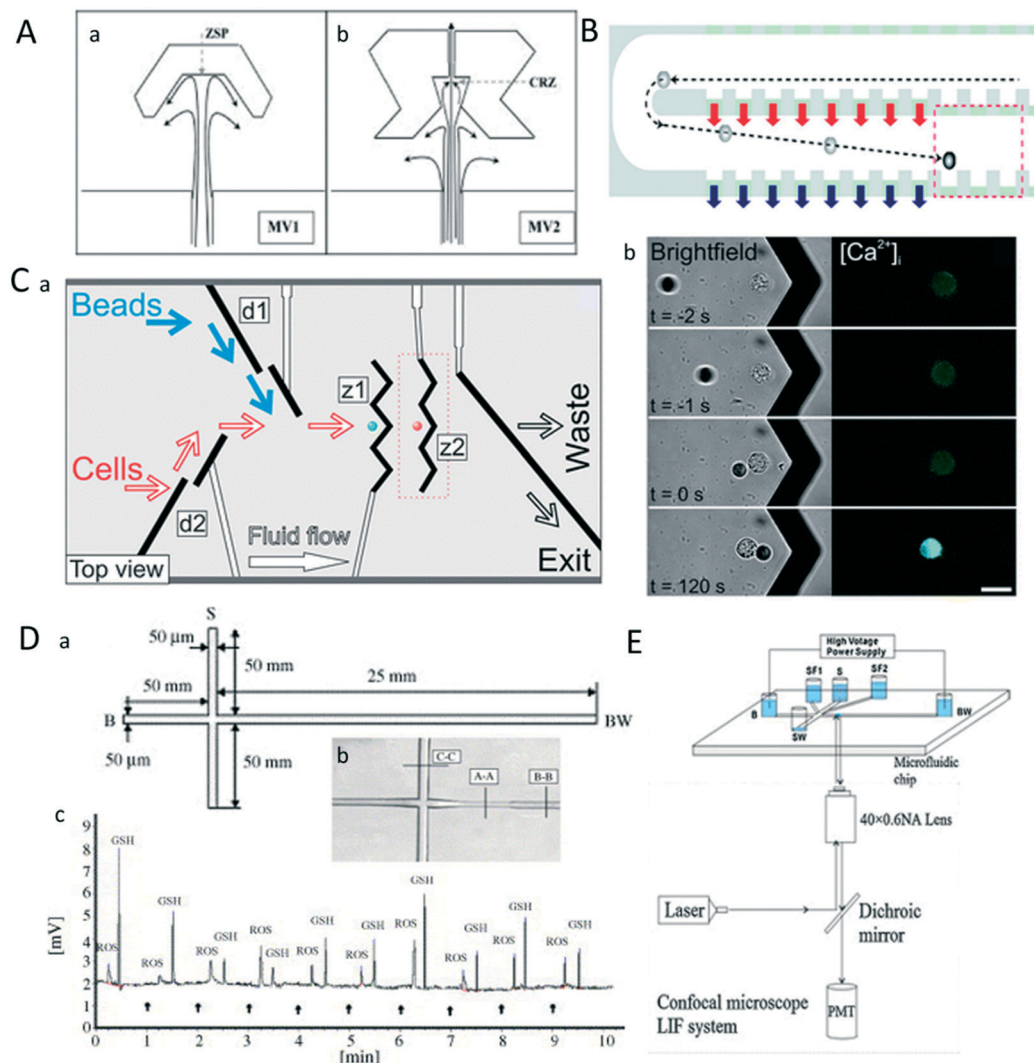
Fig. 8 A) The chip structure of MV1 (a) and MV2 (b) (reprinted from ref. 170 with permission, copyright 2005, American Chemical Society). B) Cell focusing mechanism. Cells were focused in the traps by converging flow (red arrow) and diverging flow (blue arrow) (reprinted from ref. 172 with permission, copyright 2011, American Chemical Society). C) (a) Top view of the central part of the chip. (b) Micrographs acquired at different time point shows the contact formation process between the antibody-coated beads and T cells (reprinted from ref. 174 with permission, copyright 2009, Royal Society of Chemistry). D) (a) The schematic and microscope image of the channel design of the multi-depth microchip. (b) The photograph of the multi-depth microfluidic chip. (c) The electropherogram of ROS and GSH in single HepG2 cells (reprinted from ref. 180 with permission, copyright 2006, Elsevier). E) The principle of the experimental device for simultaneous detection of ROS and GSH on microfluidic chip (reprinted from ref. 182 with permission, copyright 2011, Elsevier).

provided an excellent solution for this issue. During chip electrophoresis, a high voltage electric field is applied in the chip channels in order to drive analytes. The fluorophore-labeled molecules with different charge-to-mass ratios then go through the channel in turn, separate, and can be detected by a laser induced fluorescence device at a specific location. Hence, multiple components can be qualitatively distinguished according to the migration time of the molecules in electrophoresis and the content of each component can be quantified accurately on the basis of measured fluorescence intensity. Xue-Feng Yin *et al.*[180] designed a multi-depth microfluidic chip to trap single cells and achieved separation and quantitative detection of intracellular fluorescence derivative ROS and GSH by CE-LIF (Fig. 8D). Subse-

quently, the author used the same microchip combined with LIF technology to detect superoxide anions in single HepG2 cells.[181] Chun-Xiu Xu *et al.*[182] fabricated a cross microfluidic chip for simultaneous detection of ROS and GSH in individual erythrocytes (Fig. 8E). After derivatization of intracellular ROS and GSH by DHR 123 and NDA, the cells were introduced into the microchip and lysed, and two peaks could be generated when the fluorescence derivatives flew through the laser irradiated point. Similarly, Linfen Yu *et al.*[183] measured GSH in single K562 cells utilizing a simple cross microchip combined with a laser induced fluorescence detector.

Benefiting from the advantages of chip electrophoresis-laser induced fluorescence detection system, our group has developed a series of single-cell analytical methods for

detecting various intracellular small molecules using a home-made laser induced fluorescence detection system and laboratory-designed fluorescence probes. Initially, we exploited an electrokinetic gated injection realizing the measurement of H_2O_2 in individual HepG2 cells.[184] First, H_2O_2 in HepG2 was derived by a home-synthesized fluorescent probe, bis(p-methylbenzenesulfonate) dichlorofluorescein (FS). By switching the high voltage electric field on a simple cross chip, a series of high-efficient cell manipulation steps including single-cell injection, cytolysis, electrophoresis separation, and fluorescent detection could be accomplished. Finally, fluorescence derivatives of H_2O_2 in single PC-12 cells were accurately quantified by the laser-induced fluorescence detection (LIFD) system. Subsequently, we achieved simultaneous detection of intracellular Na^+ and K^+ derivatives, which had the same luminescence properties.[185] A novel BODIPY-based near-infrared fluorescent probe (cBDP) was designed and synthesized for simultaneous fluorescence sensing of Na^+ and K^+. Because both of them created fluorescence enhancement in the near infrared region, it is difficult to distinguish Na^+ and K^+ by fluorescent imaging. So, we employed the above developed method to realize simultaneous quantitative detection of Na^+ and K^+ in single cells based on different charge-to-mass ratios of derivatives at optimized electrophoresis conditions (Fig. 9A). What's more, we further compared the variation of Na^+ and K^+ in single normal and cancer cells, and

the alteration of intracellular Na^+ and K^+ during cell apoptosis. Although the electrokinetic gated injection method could effectively perform the manipulation of individual cells, only one cell could be analyzed during each cell injection process because the high electric field during electrophoretic separation can damage cells in the sample reservoir. To improve the throughput of single-cell analysis, we redesigned the microchip structure and developed a novel method combining hydrostatic pressure and electrokinetic gated injection, by which cells could avoid high electric field damage in sample reservoirs and be consecutively manipulated for analyses (Fig. 9B).[12] Then, intracellular $O_2^{\cdot-}$ and NO derived with DBZTC and DAF-FM DA were respectively measured by CE-LIF. Next, to adapt to wide variations in fluorescence excitation and emission spectra of multiple small molecules, we constructed a multicolor fluorescence detection-based microfluidic device (MFD-MD) to simultaneously and quantitatively determinate the concentration of H_2O_2, GSH, and Cys in single mice primary liver cells by fluorescent probes FS and Cy-3-NO_2 (Fig. 9C).[27] Then, we applied the developed multicomponent analysis method for the study of single cell metallomics. Simultaneous quantitative analysis of Na^+, K^+, Ca^{2+}, and Mg^{2+} was achieved in single neuron-like PC-12 cells (Fig. 9D).[11] Na^+ and K^+ were labeled by our homemade fluorescent probe cBDP, generating near infrared fluorescence, while Ca^{2+} and Mg^{2+} were labeled by commercial fluorescent
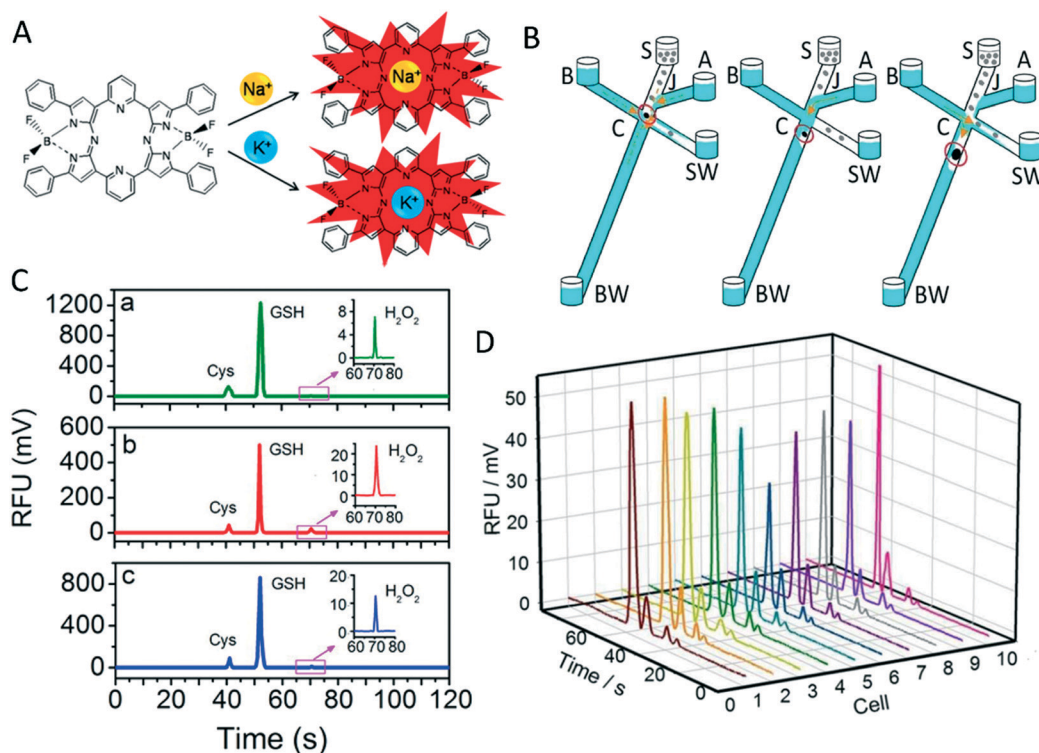

Fig. 9   A) The structure of cBDP fluorescent probe and reaction mechanism (reprinted from ref. 185 with permission, copyright 2015, American Chemical Society). B) The process of consecutive gated injection (reprinted from ref. 12 with permission, copyright 2016, American Chemical Society). C) Electropherograms of H_2O_2, Cys, and GSH in individual mice liver cells. (a) Control group; (b) stimulated with ethanol and (c) stimulated with silymarin (reprinted from ref. 27 with permission, copyright 2016, American Chemical Society). D) Electropherograms of Na^+, K^+, Ca^{2+}, and Mg^{2+} in 10 single PC-12 cells (reprinted from ref. 11 with permission, copyright 2017, American Chemical Society).

probes Fluo 3-AM and Mag-Fluo 4-AM respectively, generating green fluorescence. After capturing and lysis of single PC-12 cells on a microchip, the four intracellular derived metal ions were separated by high-efficient electrophoresis and detected quantitatively. We also studied variations of the four metal ions in established Alzheimer's disease model cells.

Except for producing fluorescence signal transformation by the specific reaction between the organic small molecular fluorescence probe and intracellular small molecules for analysis, some methods based on "always on" can be used for small molecules detection in single cells as well. For example, Jun-Tao Cao et al.[186] monitored the expression of glycans on a single K562 cell surface. Single cells were confined in microwells of a microfluidic chip. Con A was used for recognizing glycans. Then, primary antibody anti-Con A was added to recognize Con A and combined with the fluorescence labelled second antibody, QDs-IgG. Finally, glycans could be detected by fluorescence microscopy. Additionally, the author also detected sialic acid (SA) in single K562 cells on a similar microfluidic chip. The target could be specifically connected with QDs linked phenylboronic acid (PBA) by a glycol structure.[187]

3. Fluorescent devices for single-cell analysis on a microchip

Microfluidic chips, as a high effective platform for fluid control and single-cell manipulation, are always required to be integrated with extra detection technologies for target molecule detections.[179] Fluorescent methodology is quite suitable for signal collection from single cells on light-transmissive microfluidic chips, owing to it is high sensitivity and convenience for integration. There are a variety of fluorescent detection methods such as fluorescence microscopy, chip electrophoresis combined with laser induced fluorescence detection, flow cytometry, and so on. To be better compatible with a microfluidic chip for convenient and multi-parameter fluorescence detection of single-cell components, these methods are being improved and developed continuously.[26,188] An appropriate fluorescence detection method is the key to satisfy analysis requirements of different single-cells components. In this part, we will introduce single-cells fluorescent detection technologies applied for microchips in recent years from three aspects: imaging detection, chip electrophoresis combined with laser induced fluorescence detection, and flow cytometry.

3.1 Fluorescence imaging

Fluorescence microscopy has emerged as a powerful tool for visual detection of single-cells target molecules on microfluidic chips.[13] In general, laser-scanning confocal microscopy and total internal reflection fluorescence microscopy (TIRFM) are two main means for target imaging. In confocal fluorescence imaging, optically conjugated pinholes are settled behind the laser beam and in front of a detector to re-

move the background fluorescence to an out of focus position.[189] Thus, a higher signal to noise ratio can be obtained. This approach is more adaptable for the detection of living cells or immobilized cells. Except for fluorescence intensity of target molecules, more importantly, the distribution of intracellular components can also be monitored.[46,190] For instance, by virtue of this approach, Jung-uk Shim et al.[91] observed the expression of proteins mRFP1 in single bacterial cells. Ariel Kniss et al.[175] measured the fluctuation of Ca^{2+} content in single Jurket cells. Besides, total internal reflection fluorescence microscopy also has become an attractive tool with high sensitivity for cellular contents analysis, even at the single-molecule level. In TIRF detection, an evanescent wave is used to selectively excite fluorophores in a glass-liquid surface region. This can greatly eliminate the interference of background fluorescence and acquire target signals with high signal to noise ratio, so this method is well suited for low-abundance components detection in single cells. Because of a limitation of the exponential fall off of evanescent field intensity (less than 100 nanometers in thickness), TIRF is only appropriate for the determination of plasma membrane molecules or molecules released from cells that are fixed on a substrate.[191,192] Utilizing this approach, Ali Salehi-Reyhani et al.[30] detected p53 proteins in single MDA-MB-468 cell extracts; Tania Konry et al.[121] imaging analyzed membrane protein EpCAM in single PC3 cells. However, compared to the common dimension of microfluidic channels for single-cell analysis (approximately 10–100 μm width and height), the detection regions of a conventional confocal fluorescent microscope (about 500 nm wide, 1 μm high) and TIRFM (less than 100 nm in thickness) is much smaller, which results in rather low detection efficiency.[13] Hence, dimension matching between a microchannel and the focus of a fluorescence microscope is the main issue to enhance detection efficiency. There are mainly two approaches: (1) adjust the channel dimension to the focus of the confocal microscope. Nevertheless, the smaller channel may influence the electrophoretic separation efficiency and induced a microchannel block. For improving detection efficiency without clogging of the channel, Jun Wang et al.[31] designed a microchip equipped with an elastomeric valve to alter the dimension of the detection area while other locations were not changed (Fig. 10B). First, cells were aligned in a line by hydrodynamic focusing. As shown in Fig. 10B, in order to drive the cells closer to the evanescent field, the elastomeric valve was partially closed to squeeze the flowing cells to contact with the glass substrate, making cell surface fluorescent molecules able to be measured by the evanescent field generated by TIRFM. (2) Alternatively, widening the excitation laser focus by rebuilding microscope can also improve the detection efficiency. Huang et al.[13] added a cylindrical lens into the optical configuration, by which the excitation laser focus could be adjusted. In this way, laser focus was broadened greatly and its height was nearly 2 μm, which fit perfectly to the microfluidic channel (Fig. 10A). This device vastly enhanced the detection efficiency, and by utilizing it, the author
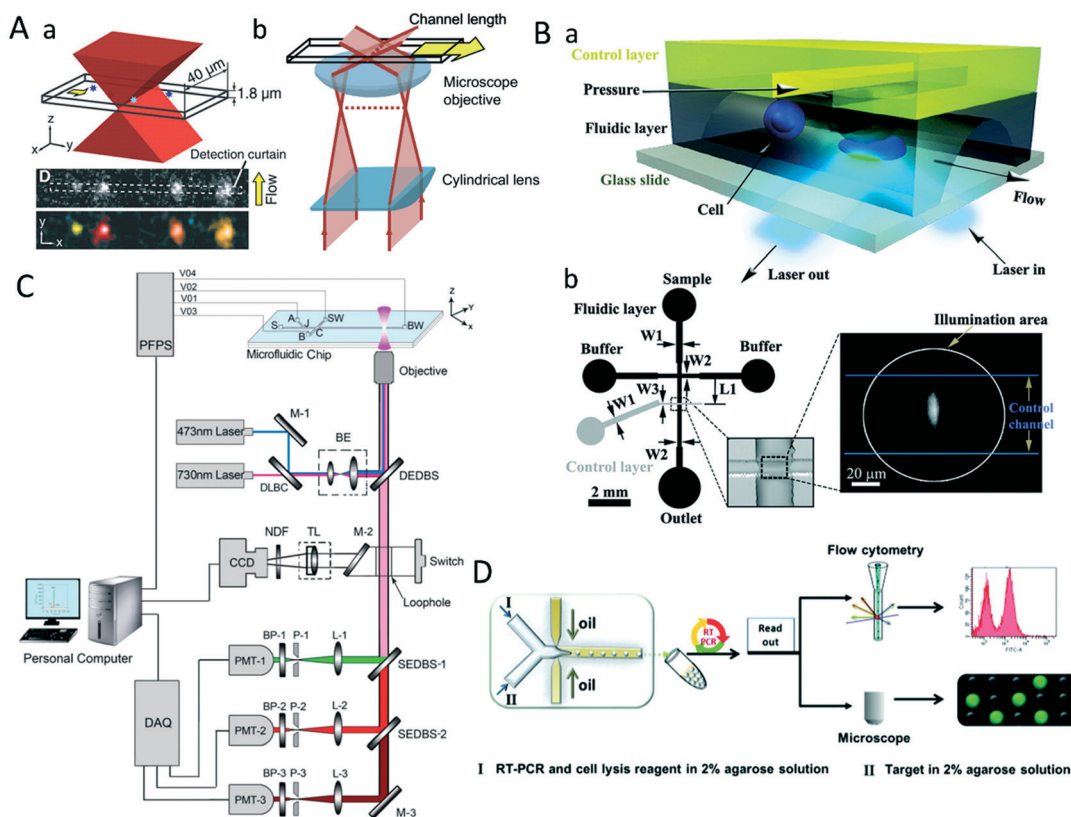
Fig. 10 A) (a) Schematic of focused light path and the image of fluorescent molecules at focal plane. (b) Schematic illustration of focused excitation light by a microscope objective (reprinted from ref. 13 with permission, copyright 2007, The American Association for the Advancement of Science). B) (a) The schematic of a microfluidic device for detecting membrane proteins. (b) Structural diagram of a microchip device and the microscope image of the illumination area (reprinted from ref. 31 with permission, copyright 2008, American Chemical Society). C) Working principle diagram of a multi-color fluorescence detection microfluidic device (reprinted from ref. 27 with permission, copyright 2016, American Chemical Society). D) Microfluidic chips produced agarose droplets for intracellular RNA detection by flow cytometry and confocal fluorescence microscopy (reprinted from ref. 66 with permission, copyright 2012, American Chemical Society).

achieved single-molecule analysis of low-copy numbers of proteins.

3.2 Chip electrophoresis – laser induced fluorescence detection (CE-LIFD)

A single cell is a quite a complex system that contains a variety of biomolecules. Hence, a great challenge single-cell components analysis is faced with is the separation and simultaneous detection of different molecules. Although simultaneously imaging detection of multi components can be realized by selecting fluorescent probes with distinguished fluorescence spectra, the limited probe types and crosstalk spectral region hampered the simultaneous analysis of multiple kinds of components. From another perspective, chip electrophoresis provides a great solution for this issue. Under the high voltage electric field applied in a microfluidic channel, fluorescent derivative of different molecules can be separated based on different charge-to-mass ratios. Coordinating with a electrophoresis separation on a microfluidic chip, different laser-induced fluorescence detection systems have been proposed to achieve simultaneous detection of multi components. Kennedy's group[193] built a laser-induced fluo-

rescence detection system utilizing an epi-fluorescence microscope integrating an Ar^+ 488 nm laser, a photon counting detector, and control and data collection software. Based on this detection system and immunoassay, they monitored insulin content secreted from single islets under glucose stimulation. Subsequently, in 2007 and 2009, they continuously investigated insulin secreted from islets using a similar detection system and method.[194,195] Xue-Feng Yin *et al.*[180] also constructed a confocal microscope LIF system using an inverted microscope coupled a 488 nm Ar^+ laser and PMT detector. ROS and GSH in single HepG2 cells were measured by this laboratory-built system. Additionally, our group developed a laser-induced fluorescence detector (LIFD) equipped with a 473 nm laser and a PMT detector, realizing H_2O_2 quantitative determination in single HepG2 cells on a cross microchip.[184] However, the existing one-laser excitation and one-color fluorescence collection system can't satisfy the simultaneous detection of multiple small molecules with wide variations in their fluorescence excitation and emission spectra.[27] Focused on this challenge, our group[27] designed and constructed a multicolor fluorescence detection-based microfluidic device (MFD-MD) for multiple constituents analysis at the single-cell level (Fig. 10C). In this system, two lasers are

used for excitation (473 nm and 730 nm) and three optics channels are used for fluorescence collection (fluorescent collection range: 515–535 nm, 745–765 nm, and 795–815 nm). Utilizing this device, we successfully quantified the concentration of H_2O_2, GSH, and Cys in single mouse primary liver cells and Na^+, K^+, Ca^{2+}, and Mg^{2+} in single neuron-like cells.[11,27]

3.3 Flow cytometry

Flow cytometry (FCM) is another effective approach using laser induced fluorescence technology for sorting and quantitative analysis of single cells. The cells are first labeled with fluorescent markers and injected into a flow cytometer. After aligned cells (or other particles) are passed through the laser detection area, the fluorescent signal is recorded by a high-sensitive detector. According to the different optical signals of each cell or particle, simultaneous multiparametric analysis of the physical and chemical characteristics of cells or particles can be accomplished. Furthermore, charges can be added to the cells or particles based on their characteristics, which is the foundation of subsequent cell sorting when they pass through the high voltage electrostatic field. This detection approach has the characteristics of fast speed, high throughput, simultaneous multicolor fluorescence detection of single-cell samples, and a sorting function triggered by the fluorescent signal. By combining with FCM, the speed and throughput of single-cell components analysis on microfluidic chips can be improved. For example, Huifa Zhang et al.,[66] using a microfluidic chip incorporating flow cytometry, detected gene expression levels of EpCAM in single cancer cells. (Fig. 10D). Similarly, Venkatachalam Chokkalingam et al.[119] detected multiple secreted proteins in single agarose droplets by a flow cytometer. In addition, after the detection of a single-cell fluorescent signal, electric field, or sound wave, etc. can be integrated with the flow cytometer for sorting desired cells;[196,197] this is fluorescence-activated cell sorting (FACS). For example, Lothar Schmid et al.[196] achieved the sorting of target cells in a complex sample by a self-designed FACS system. Mouse melanoma cells in a complex sample was first stained by calcein-AM. In the microfluidic chip, the cells aligned in a line by a co-flowing sheath flow. When the stained cells flew through the detection area, fluorescent signals were recorded by a detector. Once a desired fluorescence was detected, a surface acoustic wave would be generated to deflect this cell to the sorting channel, while cells with no fluorescence would flow into a waste reservoir. Thus, different cell sorting can be achieved according to different requirements.

Summing up the above, we concluded the researches combined microfluidic technology with the three above methods for single-cell components analysis as below (Table 1).

4. Conclusions

With vigorous development of fluorescence probes and the continuous improvement of fluorescent sensing technology, fluorescent methods have shown unique advantages and attraction in single-cell components analysis based on a micro-fluidic chip as the manipulating and detecting platform. In recent years, with continuous investigation in life sciences and higher requirement of medical diagnosis and treatment, single-cell components analysis and cell heterogeneity research have been an important research topic in the field of cytomics analysis, cell phenotyping, and precision medicine. Meanwhile, higher requirements have been proposed for the research. We think that in single-cell fluorescence analysis based on a microfluidic chip, the major challenges will be focused on fluorescent probes and sensing methods, research contents, data analysis, and information processing etc. 1, First, in the aspect of fluorescent probes and sensing methods, it is difficult to detect the original contents of intracellular biomolecules because of low abundance of some molecules in single cells. Some of the existing fluorescent probes can only respond to the amounts of the substances which are added into cells or which are elevated after stimulating the cells. Hence, sensitivity of the probes should be further improved and signal amplification methods are also required to be constantly developed to achieve single-cell analysis of the original contents of intracellular components. In addition, limited by non-reversible labelling of many fluorescent probes, the obtained signals only reflect transient molecular information when intracellular components are labeled. In order to achieve real-time analysis of intracellular components, fluorescent probes with ability of dynamic and reversible labelling are required to obtain information of spatial and temporal changes of biomolecules in cells. 2, Referring to research objects and contents as the development of omics studies (including genomics, proteomics, metabonomics and metallomics), single-cell omics study has become one of the frontier research fields. Developing novel fluorescence methods to detect multiple molecules of the same type in single cells will be important for single-cell omics studies. Additionally, in order to accurately and truly understand the molecular regulation mechanisms of various biological processes, it is necessary to study the signal pathway at the single-cells level. Aiming for the relevance and influence of different types of multiple biomolecules in one cell, how to make use of the advantages of microfluidic technologies and fluorescence methods to perform single-cell analysis is another issue urgently needed to be solved. Among above research objectives, simultaneous spatiotemporal resolution of fluorescence signals of multiple molecules in single cells will be an important challenge. Except for the detection of intracellular molecules, qualitative and quantitative analysis of molecules secreted outside cells, and monitoring spatiotemporal changes of secreted molecules among the cells is essential for the study of cell-to-cell communication. Single-cell fluorescence analysis based on a microfluidic chip can also supply significant information for cell communication research. 3, Throughput of single-cell analysis and data processing are also needed to be further improved. Although a single cell is the research subject in single-cell analysis, only statistics and analysis based on a large number of cell detection results make sense to biological and medical

Table 1 Summary of single-cell components analysis methods combined with microfluidic technology

Category	Target	Fluorescence sensing probe and method	Detection technology	Ref.
Nucleic acid	miRNAs	RT-PCR	Sequencer, epifluorescence microscopy	57, 62, 63, 81
	mRNAs	MDA, RT-PCR	Sequencer, epifluorescence microscopy	63, 66, 69–71, 198
	Others			
	rRNA	Cy5-PNA probe	Confocal microscopy,	50
	Total RNA	RT-PCR	sequencer,	60
	Extracellular matrix genes	RT-PCR	epifluorescence	61
	DNA β-actin and t(14;18)	PCR	microscopy, flow cytometry	67
Protein	Intracellular proteins (HA, GST, p53, GAPDH, AP, YFP, mRFP1, GFP)	Immunoassay with fluorophore-labeled antibody, ELISA with FDA substrate for AP	Confocal microscopy, TIRFM, epifluorescence microscopy	30, 31, 87, 91, 100, 144
	Secreted proteins (TNF-a, IFN-γ, IL-2, IL-1α, IL-1β, IL-6, IL-10, IL-12, GM-CSF, CCL-2, TGF-β, PSA)	Immunoassay with fluorophore-labeled antibody	Confocal microscopy, TIRFM, epifluorescence microscopy	89, 109, 119, 120, 122
	Membrane proteins (β2 adrenergic receptors, 5D10, CCR5, CD19, EpCAM, CD86, CD69, PTK7, GB1 receptors)	Immunoassay with fluorophore-labeled antibody, enzyme catalysis with FDG substrate for β-galactosidase, aptamer (sgc8) for PTK7, small molecule ABP probe for GB1 receptors	Confocal microscopy, epifluorescence microscopy, LIFD, flow cytometry	13, 15, 113, 121–123, 138, 174
Small molecule	Metal ions			
	Ca^{2+}	Fluo 4-AM	Confocal microscopy,	170, 171
		Fluo 3-AM	epifluorescence	172, 173
		Fura 2-AM	microscopy, LIFD	174
	Mg^{2+}	Mag-Fluo 4-AM		11
	Na^+, K^+	cBDP		11, 185
	Reactive oxygen molecules			
	Total ROS	DHR 123	Epifluorescence microscopy, CE-LIFD	176, 180, 182
	$O_2^{\cdot-}$	DHE, DBZTC		12, 181
	H_2O_2	FS		27, 184
	NO	DAF-FM		12
	Reductive small molecules			
	GSH	NDA, $Cy\text{-}3\text{-}NO_2$	Epifluorescence microscopy, LIFD	27, 176, 180, 182, 183
	Cys	$Cy\text{-}3\text{-}NO_2$		27

research. Thus, microfluidic chips are demanded to have more efficient single-cell manipulation ability and detection instruments are demanded to have faster signal capture and output abilities to ensure the improvement of single-cells detection throughput. In data processing, mathematical and statistical methods should be combined to achieve the generalization, classification, or comparison of thousands of single-cells data. These existing challenges mean new opportunities. Resolution of above issues will constantly promote the development of single-cells fluorescence analysis with higher sensitivity and higher throughput, real-time dynamic detection and multicomponent analysis, and finally promote a more accurate and comprehensive understanding of the regulation mechanism of intracellular molecules in biological processes, thus providing new methods and information for research and development in biology and medicine fields.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Notes and references

1 G. Bao and S. Suresh, *Nat. Mater.*, 2003, 2, 715–725.
2 A. A. Pollen, T. J. Nowakowski, J. Shuga, X. Wang, A. A. Leyrat, J. H. Lui, N. Li, L. Szpankowski, B. Fowler, P. Chen, N. Ramalingam, G. Sun, M. Thu, M. Norris, R. Lebofsky, D. Toppani, D. W. Kemp Ii, M. Wong, B. Clerkson, B. N. Jones, S. Wu, L. Knutsson, B. Alvarado, J. Wang, L. S. Weaver, A. P. May, R. C. Jones, M. A. Unger, A. R. Kriegstein and J. A. A. West, *Nat. Biotechnol.*, 2014, 32, 1053–1058.
3 M. B. Elowitz, A. J. Levine, E. D. Siggia and P. S. Swain, *Science*, 2002, 297, 1183–1186.

4 R. H. Templer and O. Ces, *J. R. Soc., Interface*, 2008, **5**, S111–S112.

5 O. Brandman, J. E. Ferrell, R. Li and T. Meyer, *Science*, 2005, **310**, 496–498.

6 X. Mu, W. Zheng, J. Sun, W. Zhang and X. Jiang, *Small*, 2013, **9**, 9–21.

7 S. Lindstrom and H. Andersson-Svahn, *Lab Chip*, 2010, **10**, 3363–3372.

8 G. T. Roman, Y. Chen, P. Viberg, A. H. Culbertson and C. T. Culbertson, *Anal. Bioanal. Chem.*, 2007, **387**, 9–12.

9 M. Junkin and S. Tay, *Lab Chip*, 2014, **14**, 1246–1260.

10 H. N. Joensson and H. Andersson Svahn, *Angew. Chem., Int. Ed.*, 2012, **51**, 12176–12192.

11 L. Li, Y. Fan, Q. Li, R. Sheng, H. Si, J. Fang, L. Tong and B. Tang, *Anal. Chem.*, 2017, **89**, 4559–4565.

12 L. Li, Q. Li, P. Chen, Z. Li, Z. Chen and B. Tang, *Anal. Chem.*, 2016, **88**, 930–936.

13 B. Huang, H. Wu, D. Bhaya, A. Grossman, S. Granier, B. K. Kobilka and R. N. Zare, *Science*, 2007, **315**, 81–84.

14 L. Shui, S. Pennathur, J. C. T. Eijkel and A. van den Berg, *Lab Chip*, 2008, **8**, 1010–1014.

15 L. Li, Q. Wang, J. Feng, L. Tong and B. Tang, *Anal. Chem.*, 2014, **86**, 5101–5107.

16 Y. Xie, X. Zhi, H. Su, K. Wang, Z. Yan, N. He, J. Zhang, D. Chen and D. Cui, *Nanoscale Res. Lett.*, 2015, **10**, 477.

17 B. Wang, H. Zhou, Y. Luo, R. Tang and S. Zheng, *Curr. Med. Chem.*, 2014, **21**, 2510–2521.

18 M. Poudineh, P. M. Aldridge, S. Ahmed, B. J. Green, L. Kermanshah, V. Nguyen, C. Tu, R. M. Mohamadi, R. K. Nam, A. Hansen, S. S. Sridhar, A. Finelli, N. E. Fleshner, A. M. Joshua, E. H. Sargent and S. O. Kelley, *Nat. Nanotechnol.*, 2017, **12**, 274–281.

19 Y.-C. Tung, N.-T. Huang, B.-R. Oh, B. Patra, C.-C. Pan, T. Qiu, P. K. Chu, W. Zhang and K. Kurabayashi, *Lab Chip*, 2012, **12**, 3552–3565.

20 N.-T. Huang, H.-L. Zhang, M.-T. Chung, J. H. Seo and K. Kurabayashi, *Lab Chip*, 2014, **14**, 1230–1245.

21 C. E. Sims and N. L. Allbritton, *Lab Chip*, 2007, **7**, 423–440.

22 J. Avesar, T. B. Arye and S. Levenberg, *Lab Chip*, 2014, **14**, 2161–2167.

23 S. S. Aw, M. X. M. Tang, Y. N. Teo and S. M. Cohen, *Nucleic Acids Res.*, 2016, **44**, e92.

24 F. Ma, Y. Li, B. Tang and C.-Y. Zhang, *Acc. Chem. Res.*, 2016, **49**, 1722–1730.

25 W. Gao, Y. Zhao, X. Li, Y. Sun, M. Cai, W. Cao, Z. Liu, L. Tong, G. Cui and B. Tang, *Chem. Sci.*, 2018, **9**, 439–445.

26 G. Ryu, J. Huang, O. Hofmann, C. A. Walshe, J. Y. Y. Sze, G. D. McClean, A. Mosley, S. J. Rattle, J. C. deMello, A. J. deMello and D. D. C. Bradley, *Lab Chip*, 2011, **11**, 1664–1670.

27 Q. Li, P. Chen, Y. Fan, X. Wang, K. Xu, L. Li and B. Tang, *Anal. Chem.*, 2016, **88**, 8610–8616.

28 M. Luan, N. Li, W. Pan, L. Yang, Z. Yu and B. Tang, *Chem. Commun.*, 2017, **53**, 356–359.

29 L. Yang, Y. Chen, W. Pan, H. Wang, N. Li and B. Tang, *Anal. Chem.*, 2017, **89**, 6196–6201.

30 A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, M. Novakova, A. J. deMello, R. H. Templer, P. Parker, M. A. A. Neil, O. Ces, P. French, K. R. Willison and D. Klug, *Lab Chip*, 2011, **11**, 1256–1261.

31 J. Wang, N. Bao, L. L. Paris, R. L. Geahlen and C. Lu, *Anal. Chem.*, 2008, **80**, 9840–9844.

32 D. R. Bandura, V. I. Baranov, O. I. Ornatsky, A. Antonov, R. Kinach, X. Lou, S. Pavlov, S. Vorobiev, J. E. Dick and S. D. Tanner, *Anal. Chem.*, 2009, **81**, 6813–6822.

33 K. Czechowska, D. R. Johnson and J. R. van der Meer, *Curr. Opin. Microbiol.*, 2008, **11**, 205–212.

34 H. Andersson and A. van den Berg, *Curr. Opin. Biotechnol.*, 2004, **15**, 44–49.

35 J. D. Watson and F. H. C. Crick, *Nature*, 1953, **171**, 737–738.

36 Y. Zhao, F. Chen, Q. Li, L. Wang and C. Fan, *Chem. Rev.*, 2015, **115**, 12491–12545.

37 L. F. Cavalieri, *J. Cell. Comp. Physiol.*, 1963, **62**, 111–122.

38 L. Salmon, S. Yang and H. M. Al-Hashimi, *Annu. Rev. Phys. Chem.*, 2014, **65**, 293–316.

39 H. Sun, J. Sheng, A. E. A. Hassan, S. Jiang, J. Gan and Z. Huang, *Nucleic Acids Res.*, 2012, **40**, 5171–5179.

40 J. M. Ogle, D. E. Brodersen, W. M. Clemons, M. J. Tarry, A. P. Carter and V. Ramakrishnan, *Science*, 2001, **292**, 897–902.

41 Y. Liu, A. Beyer and R. Aebersold, *Cell*, 2016, **165**, 535–550.

42 K. H. Chen, A. N. Boettiger, J. R. Moffitt, S. Wang and X. Zhuang, *Science*, 2015, **348**, aaa6090.

43 T. Suzuki, M. Terasaki, C. Takemoto-Hori, T. Hanada, T. Ueda, A. Wada and K. Watanabe, *J. Biol. Chem.*, 2001, **276**, 21724–21736.

44 S. Yekta, I. h. Shih and D. P. Bartel, *Science*, 2004, **304**, 594.

45 M. Selbach, B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin and N. Rajewsky, *Nature*, 2008, **455**, 58–63.

46 R. Deng, L. Tang, Q. Tian, Y. Wang, L. Lin and J. Li, *Angew. Chem., Int. Ed.*, 2014, **53**, 2389–2393.

47 J. Michaelis, A. Roloff and O. Seitz, *Org. Biomol. Chem.*, 2014, **12**, 2821–2833.

48 J. M. Levsky and R. H. Singer, *J. Cell Sci.*, 2003, **116**, 2833–2838.

49 R. Amann and B. M. Fuchs, *Nat. Rev. Microbiol.*, 2008, **6**, 339–348.

50 T. D. Rane, H. C. Zec, C. Puleo, A. P. Lee and T.-H. Wang, *Lab Chip*, 2012, **12**, 3341–3347.

51 H. Deng and Z. Gao, *Anal. Chim. Acta*, 2015, **853**, 30–45.

52 T. Notomi, Y. Mori, N. Tomita and H. Kanda, *J. Microbiol.*, 2015, **53**, 1–5.

53 L. Mazutis, A. F. Araghi, O. J. Miller, J.-C. Baret, L. Frenz, A. Janoshazi, V. Taly, B. J. Miller, J. B. Hutchison, D. Link, A. D. Griffiths and M. Ryckelynck, *Anal. Chem.*, 2009, **81**, 4813–4821.

54 L. Li, J. Feng, H. Liu, Q. Li, L. Tong and B. Tang, *Chem. Sci.*, 2016, **7**, 1940–1945.

55 H. Liu, L. Li, Q. Wang, L. Duan and B. Tang, *Anal. Chem.*, 2014, **86**, 5487–5493.

56 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich and N. Arnheim, *Science*, 1985, **230**, 1350–1354.

57 H. Tian, Y. Sun, C. Liu, X. Duan, W. Tang and Z. Li, *Anal. Chem.*, 2016, **88**, 11384–11389.

58 W. J. Allard, J. Matera, M. C. Miller, M. Repollet, M. C. Connelly, C. Rao, A. G. J. Tibbe, J. W. Uhr and L. W. M. M. Terstappen, *Clin. Cancer Res.*, 2004, **10**, 6897–6904.

59 E. Ozkumur, A. M. Shah, J. C. Ciciliano, B. L. Emmink, D. T. Miyamoto, E. Brachtel, M. Yu, P.-I. Chen, B. Morgan, J. Trautwein, A. Kimura, S. Sengupta, S. L. Stott, N. M. Karabacak, T. A. Barber, J. R. Walsh, K. Smith, P. S. Spuhler, J. P. Sullivan, R. J. Lee, D. T. Ting, X. Luo, A. T. Shaw, A. Bardia, L. V. Sequist, D. N. Louis, S. Maheswaran, R. Kapur, D. A. Haber and M. Toner, *Sci. Transl. Med.*, 2013, **5**, 179ra147.

60 D. T. Miyamoto, Y. Zheng, B. S. Wittner, R. J. Lee, H. Zhu, K. T. Broderick, R. Desai, D. B. Fox, B. W. Brannigan, J. Trautwein, K. S. Arora, N. Desai, D. M. Dahl, L. V. Sequist, M. R. Smith, R. Kapur, C.-L. Wu, T. Shioda, S. Ramaswamy, D. T. Ting, M. Toner, S. Maheswaran and D. A. Haber, *Science*, 2015, **349**, 1351–1356.

61 D. T. Ting, B. S. Wittner, M. Ligorio, N. Vincent Jordan, A. M. Shah, D. T. Miyamoto, N. Aceto, F. Bersani, B. W. Brannigan, K. Xega, J. C. Ciciliano, H. Zhu, O. C. MacKenzie, J. Trautwein, K. S. Arora, M. Shahid, H. L. Ellis, N. Qu, N. Bardeesy, M. N. Rivera, V. Deshpande, C. R. Ferrone, R. Kapur, S. Ramaswamy, T. Shioda, M. Toner, S. Maheswaran and D. A. Haber, *Cell Rep.*, 2014, **8**, 1905–1918.

62 A. K. White, M. VanInsberghe, O. I. Petriv, M. Hamidi, D. Sikorski, M. A. Marra, J. Piret, S. Aparicio and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13999–14004.

63 A. K. White, K. A. Heyries, C. Doolin, M. VanInsberghe and C. L. Hansen, *Anal. Chem.*, 2013, **85**, 7182–7190.

64 D.-K. Kang, M. Monsur Ali, K. Zhang, E. J. Pone and W. Zhao, *TrAC, Trends Anal. Chem.*, 2014, **58**, 145–153.

65 D. J. Eastburn, A. Sciambi and A. R. Abate, *Anal. Chem.*, 2013, **85**, 8016–8021.

66 H. Zhang, G. Jenkins, Y. Zou, Z. Zhu and C. J. Yang, *Anal. Chem.*, 2012, **84**, 3599–3606.

67 R. Novak, Y. Zeng, J. Shuga, G. Venugopalan, D. A. Fletcher, M. T. Smith and R. A. Mathies, *Angew. Chem., Int. Ed.*, 2011, **50**, 390–395.

68 S. Islam, A. Zeisel, S. Joost, G. La Manno, P. Zajac, M. Kasper, P. Lonnerberg and S. Linnarsson, *Nat. Methods*, 2014, **11**, 163–166.

69 E. Z. Macosko, A. Basu, R. Satija, J. Nemesh, K. Shekhar, M. Goldman, I. Tirosh, A. R. Bialas, N. Kamitaki, E. M. Martersteck, J. J. Trombetta, D. A. Weitz, J. R. Sanes, A. K. Shalek, A. Regev and S. A. McCarroll, *Cell*, 2015, **161**, 1202–1214.

70 K. Shekhar, S. W. Lapan, I. E. Whitney, N. M. Tran, E. Z. Macosko, M. Kowalczyk, X. Adiconis, J. Z. Levin, J. Nemesh, M. Goldman, S. A. McCarroll, C. L. Cepko, A. Regev and J. R. Sanes, *Cell*, 2016, **166**, 1308–1323, e1330.

71 R. Zilionis, J. Nainys, A. Veres, V. Savova, D. Zemmour, A. M. Klein and L. Mazutis, *Nat. Protoc.*, 2017, **12**, 44–73.

72 A. N. Analytical Methods Committee, *Anal. Methods*, 2014, **6**, 333–336.

73 A. Fire and S. Q. Xu, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 4641–4645.

74 S. A. Khan, *Microbiol. Mol. Biol. Rev.*, 1997, **61**, 442–455.

75 P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas and D. C. Ward, *Nat. Genet.*, 1998, **19**, 225–232.

76 A. Cao and C.-Y. Zhang, *Anal. Chem.*, 2012, **84**, 6199–6205.

77 Q. Tian, Y. Wang, R. Deng, L. Lin, Y. Liu and J. Li, *Nanoscale*, 2015, **7**, 987–993.

78 H. Liu, L. Li, L. Duan, X. Wang, Y. Xie, L. Tong, Q. Wang and B. Tang, *Anal. Chem.*, 2013, **85**, 7941–7947.

79 M. Wu, M. Piccini, C.-Y. Koh, K. S. Lam and A. K. Singh, *PLoS One*, 2013, **8**, e55044.

80 C. Spits, C. Le Caignec, M. De Rycke, L. Van Haute, A. Van Steirteghem, I. Liebaers and K. Sermon, *Nat. Protoc.*, 2006, **1**, 1965–1970.

81 L. Blanco, A. Bernad, J. M. Lázaro, G. Martín, C. Garmendia and M. Salas, *J. Biol. Chem.*, 1989, **264**, 8935–8940.

82 J. M. Lage, J. H. Leamon, T. Pejovic, S. Hamann, M. Lacey, D. Dillon, R. Segraves, B. Vossbrinck, A. González, D. Pinkel, D. G. Albertson, J. Costa and P. M. Lizardi, *Genome Res.*, 2003, **13**, 294–307.

83 Y. Fu, C. Li, S. Lu, W. Zhou, F. Tang, X. S. Xie and Y. Huang, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 11923–11928.

84 D. Eisenberg, E. M. Marcotte, I. Xenarios and T. O. Yeates, *Nature*, 2000, **405**, 823–826.

85 W. Gao, Y. Sun, M. Cai, Y. Zhao, W. Cao, Z. Liu, G. Cui and B. Tang, *Nat. Commun.*, 2018, **9**, 231.

86 P. E. Wright and H. J. Dyson, *J. Mol. Biol.*, 1999, **293**, 321–331.

87 A. Huebner, M. Srisa-Art, D. Holt, C. Abell, F. Hollfelder, A. J. deMello and J. B. Edel, *Chem. Commun.*, 2007, 1218–1220, DOI: 10.1039/B618570C.

88 J. S. Lee, H.-A. Joung, M.-G. Kim and C. B. Park, *ACS Nano*, 2012, **6**, 2978–2983.

89 C. Ma, R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R. C. Koya, C.-C. Liu, G. A. Kwong, C. G. Radu, A. Ribas and J. R. Heath, *Nat. Med.*, 2011, **17**, 738–743.

90 K. R. MacKenzie, *Chem. Rev.*, 2006, **106**, 1931–1977.

91 J.-U. Shim, L. F. Olguin, G. Whyte, D. Scott, A. Babtie, C. Abell, W. T. S. Huck and F. Hollfelder, *J. Am. Chem. Soc.*, 2009, **131**, 15251–15256.

92 S. Dallongeville, N. Garnier, C. Rolando and C. Tokarski, *Chem. Rev.*, 2016, **116**, 2–79.

93 K. Kuningas, H. Päkkilä, T. Ukonaho, T. Rantanen, T. Lövgren and T. Soukka, *Clin. Chem.*, 2007, **53**, 145.

94 S. Kim, E. Geiss, P. Yu, F. Papadimitrakopoulos and H. L. Marcus, *Mater. Sci. Eng., C*, 2006, **26**, 1401–1407.

95 S. Wennmalm, L. Edman and R. Rigler, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, **94**, 10641–10646.

96 G. Festag, A. Steinbrück, A. Wolff, A. Csaki, R. Möller and W. Fritzsche, *J. Fluoresc.*, 2005, **15**, 161–170.

97 J. B. Lamture, K. Lbeattie, B. E. Burke, M. D. Eggers, D. J. Ehrlich, R. Fowler, M. A. Hollis, B. B. Kosicki, R. K. Reich, S. R. Smith, R. S. Varma and M. E. Hogan, *Nucleic Acids Res.*, 1994, **22**, 2121–2125.

98 L. Edman, Z. Földes-Papp, S. Wennmalm and R. Rigler, *Chem. Phys.*, 1999, **247**, 11–22.

99 D. J. Kinahan, S. M. Kearney, N. Dimov, M. T. Glynn and J. Ducree, *Lab Chip*, 2014, **14**, 2249–2258.

100 K. Eyer, S. Stratz, P. Kuhn, S. K. Küster and P. S. Dittrich, *Anal. Chem.*, 2013, **85**, 3280–3287.

101 Y. Sasuga, T. Iwasawa, K. Terada, Y. Oe, H. Sorimachi, O. Ohara and Y. Harada, *Anal. Chem.*, 2008, **80**, 9141–9149.

102 M. Stastna and J. E. Van Eyk, *Proteomics*, 2012, **12**, 722–735.

103 J. Lippincott-Schwartz, T. H. Roberts and K. Hirschberg, *Annu. Rev. Cell Dev. Biol.*, 2000, **16**, 557–589.

104 R. B. Freedman, *Cell*, 1989, **57**, 1069–1072.

105 M. E. Trujillo and P. E. Scherer, *J. Intern. Med.*, 2005, **257**, 167–175.

106 S. Doroudgar and C. C. Glembotski, *Trends Mol. Med.*, 2011, **17**, 207–214.

107 S. Shao and R. S. Hegde, *Cell*, 2011, **147**, 1576–1588.

108 W. Nickel, *Curr. Opin. Biotechnol.*, 2010, **21**, 621–626.

109 D.-S. Shin, J. You, A. Rahimian, T. Vu, C. Siltanen, A. Ehsanipour, G. Stybayeva, J. Sutcliffe and A. Revzin, *Angew. Chem., Int. Ed.*, 2014, **53**, 8221–8224.

110 Y. Zhang, Y. Tang, S. Sun, Z. Wang, W. Wu, X. Zhao, D. M. Czajkowsky, Y. Li, J. Tian, L. Xu, W. Wei, Y. Deng and Q. Shi, *Anal. Chem.*, 2015, **87**, 9761–9768.

111 E. C. Schirmer, L. Florens, T. Guan, J. R. Yates and L. Gerace, *Science*, 2003, **301**, 1380–1382.

112 I. Bahar, T. R. Lezon, A. Bakan and I. H. Shrivastava, *Chem. Rev.*, 2010, **110**, 1463–1497.

113 V. Sivagnanam, B. Song, C. Vandevyver, J.-C. G. Bünzli and M. A. M. Gijs, *Langmuir*, 2010, **26**, 6091–6096.

114 L. Shang, Y. Cheng and Y. Zhao, *Chem. Rev.*, 2017, **117**, 7964–8040.

115 S.-Y. Park, T.-H. Wu, Y. Chen, M. A. Teitell and P.-Y. Chiou, *Lab Chip*, 2011, **11**, 1010–1012.

116 H.-H. Jeong, V. R. Yelleswarapu, S. Yadavali, D. Issadore and D. Lee, *Lab Chip*, 2015, **15**, 4387–4392.

117 R. Arayanarakool, L. Shui, S. W. M. Kengen, A. van den Berg and J. C. T. Eijkel, *Lab Chip*, 2013, **13**, 1955–1962.

118 J. Wang, M. Jin, Y. Gong, H. Li, S. Wu, Z. Zhang, G. Zhou, L. Shui, J. C. T. Eijkel and A. van den Berg, *Lab Chip*, 2017, **17**, 1970–1979.

119 V. Chokkalingam, J. Tel, F. Wimmers, X. Liu, S. Semenov, J. Thiele, C. G. Figdor and W. T. S. Huck, *Lab Chip*, 2013, **13**, 4740–4744.

120 L. Mazutis, J. Gilbert, W. L. Ung, D. A. Weitz, A. D. Griffiths and J. A. Heyman, *Nat. Protoc.*, 2013, **8**, 870–891.

121 T. Konry, I. Smolina, J. M. Yarmush, D. Irimia and M. L. Yarmush, *Small*, 2011, **7**, 395–400.

122 T. Konry, A. Golberg and M. Yarmush, *Sci. Rep.*, 2013, **3**, 3179.

123 H. N. Joensson, M. L. Samuels, E. R. Brouzes, M. Medkova, M. Uhlén, D. R. Link and H. Andersson-Svahn, *Angew. Chem., Int. Ed.*, 2009, **48**, 2518–2521.

124 T. Hermann and D. J. Patel, *Science*, 2000, **287**, 820–825.

125 D. Shangguan, Y. Li, Z. Tang, Z. C. Cao, H. W. Chen, P. Mallikaratchy, K. Sefah, C. J. Yang and W. Tan, *Proc. Natl. Acad. Sci. U. S. A.*, 2006, **103**, 11838–11843.

126 G. Zhou, G. Wilson, L. Hebbard, W. Duan, C. Liddle, J. George and L. Qiao, *Oncotarget*, 2016, **7**, 13446–13463.

127 A. D. Keefe, S. Pai and A. Ellington, *Nat. Rev. Drug Discovery*, 2010, **9**, 537–550.

128 K. Sefah, D. Shangguan, X. Xiong, M. B. O'Donoghue and W. Tan, *Nat. Protoc.*, 2010, **5**, 1169–1185.

129 W. Tan, M. J. Donovan and J. Jiang, *Chem. Rev.*, 2013, **113**, 2842–2862.

130 J. M. Jackson, M. A. Witek, J. W. Kamande and S. A. Soper, *Chem. Soc. Rev.*, 2017, **46**, 4245–4280.

131 Y. Song, T. Tian, Y. Shi, W. Liu, Y. Zou, T. Khajvand, S. Wang, Z. Zhu and C. Yang, *Chem Sci*, 2017, **8**, 1736–1751.

132 J. Song, F. Lv, G. Yang, L. Liu, Q. Yang and S. Wang, *Chem. Commun.*, 2012, **48**, 7465–7467.

133 P. He, L. Liu, W. Qiao and S. Zhang, *Chem. Commun.*, 2014, **50**, 1481–1484.

134 J. Chan, S. C. Dodani and C. J. Chang, *Nat. Chem.*, 2012, **4**, 973–984.

135 L. M. Wysocki and L. D. Lavis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 752–759.

136 S. Wang, N. Li, W. Pan and B. Tang, *TrAC, Trends Anal. Chem.*, 2012, **39**, 3–37.

137 S. Mizukami, Y. Hori and K. Kikuchi, *Acc. Chem. Res.*, 2014, **47**, 247–256.

138 F. Xu, H. Zhao, X. Feng, L. Chen, D. Chen, Y. Zhang, F. Nan, J. Liu and B.-F. Liu, *Angew. Chem.*, 2014, **126**, 6848–6851.

139 E. C. Metto, K. Evans, P. Barney, A. H. Culbertson, D. B. Gunasekara, G. Caruso, M. K. Hulvey, J. A. Fracassi da Silva, S. M. Lunte and C. T. Culbertson, *Anal. Chem.*, 2013, **85**, 10188–10195.

140 S.-R. Ryoo, Y.-K. Kim, M.-H. Kim and D.-H. Min, *ACS Nano*, 2010, **4**, 6587–6598.

141 I. I. Slowing, J. L. Vivero-Escoto, C.-W. Wu and V. S. Y. Lin, *Adv. Drug Delivery Rev.*, 2008, **60**, 1278–1288.

142 M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher, *Science*, 1994, **263**, 802–805.

143 R. Rizzuto, M. Brini, P. Pizzo, M. Murgia and T. Pozzan, *Curr. Biol.*, 1995, **5**, 635–642.

144 M. C. Park, J. Y. Hur, H. S. Cho, S.-H. Park and K. Y. Suh, *Lab Chip*, 2011, **11**, 79–86.

145 A. B. Aurora, A. I. Mahmoud, X. Luo, B. A. Johnson, E. van Rooij, S. Matsuzaki, K. M. Humphries, J. A. Hill, R. Bassel-Duby, H. A. Sadek and E. N. Olson, *J. Clin. Invest.*, 2012, **122**, 1222–1232.

146 A. R. Taylor, A. Chrachri, G. Wheeler, H. Goddard and C. Brownlee, *PLoS Biol.*, 2011, **9**, e1001085.

147 R. Wang, *Physiol. Rev.*, 2012, **92**, 791–896.

148 G. Caliendo, G. Cirino, V. Santagada and J. L. Wallace, *J. Med. Chem.*, 2010, **53**, 6275–6286.

149 J. Chen, K. Wang, L. Hartman and W. Zhou, *J. Phys. Chem. A*, 2008, **112**, 16017–16021.

150 H. A. Henthorn and M. D. Pluth, *J. Am. Chem. Soc.*, 2015, **137**, 15330–15336.

151 K. Xu, M. Qiang, W. Gao, R. Su, N. Li, Y. Gao, Y. Xie, F. Kong and B. Tang, *Chem. Sci.*, 2013, **4**, 1079–1086.

152 F. Kong, L. Ge, X. Pan, K. Xu, X. Liu and B. Tang, *Chem. Sci.*, 2016, **7**, 1051–1056.

153 X. Xie, X. E. Yang, T. Wu, Y. Li, M. Li, Q. Tan, X. Wang and B. Tang, *Anal. Chem.*, 2016, **88**, 8019–8025.

154 X. Wang, J. Sun, W. Zhang, X. Ma, J. Lv and B. Tang, *Chem. Sci.*, 2013, **4**, 2551–2556.

155 P. Li, L. Fang, H. Zhou, W. Zhang, X. Wang, N. Li, H. Zhong and B. Tang, *Chem. – Eur. J.*, 2011, **17**, 10520–10523.

156 P. Li, W. Zhang, K. Li, X. Liu, H. Xiao, W. Zhang and B. Tang, *Anal. Chem.*, 2013, **85**, 9877–9881.

157 H. Xiao, P. Li, W. Zhang and B. Tang, *Chem. Sci.*, 2016, **7**, 1588–1593.

158 P. Li, H. Xiao, Y. Cheng, W. Zhang, F. Huang, W. Zhang, H. Wang and B. Tang, *Chem. Commun.*, 2014, **50**, 7184–7187.

159 P. Li, X. Duan, Z. Chen, Y. Liu, T. Xie, L. Fang, X. Li, M. Yin and B. Tang, *Chem. Commun.*, 2011, **47**, 7755–7757.

160 W. Zhang, P. Li, F. Yang, X. Hu, C. Sun, W. Zhang, D. Chen and B. Tang, *J. Am. Chem. Soc.*, 2013, **135**, 14956–14959.

161 B. Tang, Y. Xing, P. Li, N. Zhang, F. Yu and G. Yang, *J. Am. Chem. Soc.*, 2007, **129**, 11666–11667.

162 X. Jiao, Y. Li, J. Niu, X. Xie, X. Wang and B. Tang, *Anal. Chem.*, 2018, **90**, 533–555.

163 B. Tang, F. Yu, P. Li, L. Tong, X. Duan, T. Xie and X. Wang, *J. Am. Chem. Soc.*, 2009, **131**, 3016–3023.

164 S. K. Yang, X. Shi, S. Park, T. Ha and S. C. Zimmerman, *Nat. Chem.*, 2013, **5**, 692–697.

165 D. E. Clapham, *Cell*, 1995, **80**, 259–268.

166 M. J. Berridge, M. D. Bootman and P. Lipp, *Nature*, 1998, **395**, 645–648.

167 T. A. Springer, H. Jing and J. Takagi, *Cell*, 2000, **102**, 275–277.

168 W. Yang, L. M. Jones, L. Isley, Y. Ye, H.-W. Lee, A. Wilkins, Z.-R. Liu, H. W. Hellinga, R. Malchow, M. Ghazi and J. J. Yang, *J. Am. Chem. Soc.*, 2003, **125**, 6165–6171.

169 H. Komatsu, T. Miki, D. Citterio, T. Kubota, Y. Shindo, Y. Kitamura, K. Oka and K. Suzuki, *J. Am. Chem. Soc.*, 2005, **127**, 10798–10799.

170 X. Li and P. C. H. Li, *Anal. Chem.*, 2005, **77**, 4315–4322.

171 X. Li, J. Huang, G. F. Tibbits and P. C. H. Li, *Electrophoresis*, 2007, **28**, 4723–4733.

172 K. Chung, C. A. Rivet, M. L. Kemp and H. Lu, *Anal. Chem.*, 2011, **83**, 7044–7052.

173 L. Chingozha, M. Zhan, C. Zhu and H. Lu, *Anal. Chem.*, 2014, **86**, 10138–10147.

174 M. Kirschbaum, M. S. Jaeger and C. Duschl, *Lab Chip*, 2009, **9**, 3517–3525.

175 A. Kniss, H. Lu, D. P. Jones and M. L. Kemp, *Methods Enzymol.*, 2013, **526**, 219–230.

176 N. Gao, L. Li, Z. Shi, X. Zhang and W. Jin, *Electrophoresis*, 2007, **28**, 3966–3975.

177 W. Pan, H. Yang, N. Li, L. Yang and B. Tang, *Chem. – Eur. J.*, 2015, **21**, 6070–6073.

178 N. Li, C. Chang, W. Pan and B. Tang, *Angew. Chem., Int. Ed.*, 2012, **51**, 7426–7430.

179 R. N. Zare and S. Kim, *Annu. Rev. Biomed. Eng.*, 2010, **12**, 187–201.

180 S. Yue and Y. Xue-Feng, *J. Chromatogr. A*, 2006, **1117**, 228–233.

181 L. Zhu, M. Lu and X. Yin, *Talanta*, 2008, **75**, 1227–1233.

182 C.-X. Xu and X.-F. Yin, *J. Chromatogr. A*, 2011, **1218**, 726–732.

183 L. Yu, H. Huang, X. Dong, D. Wu, J. Qin and B. Lin, *Electrophoresis*, 2008, **29**, 5055–5060.

184 X. Zhang, Q. Li, Z. Chen, H. Li, K. Xu, L. Zhang and B. Tang, *Lab Chip*, 2011, **11**, 1144–1150.

185 L. Li, P. Li, J. Fang, Q. Li, H. Xiao, H. Zhou and B. Tang, *Anal. Chem.*, 2015, **87**, 6057–6063.

186 J.-T. Cao, Z.-X. Chen, X.-Y. Hao, P.-H. Zhang and J.-J. Zhu, *Anal. Chem.*, 2012, **84**, 10097–10104.

187 J.-T. Cao, P.-H. Zhang, Y.-M. Liu, E. S. Abdel-Halim and J.-J. Zhu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 14878–14884.

188 M. L. Chabinyc, D. T. Chiu, J. C. McDonald, A. D. Stroock, J. F. Christian, A. M. Karger and G. M. Whitesides, *Anal. Chem.*, 2001, **73**, 4491–4498.

189 R. Wannemacher, in *Encyclopedia of Nanotechnology*, ed. B. Bhushan, Springer Netherlands, Dordrecht, 2016, pp. 673–691.

190 R. Deng, K. Zhang, Y. Sun, X. Ren and J. Li, *Chem. Sci.*, 2017, **8**, 3668–3675.

191 Y. Sako, S. Minoghchi and T. Yanagida, *Nat. Cell Biol.*, 2000, **2**, 168–172.

192 D. Axelrod, *J. Cell Biol.*, 1981, **89**, 141.

193 J. G. Shackman, G. M. Dahlgren, J. L. Peters and R. T. Kennedy, *Lab Chip*, 2005, **5**, 56–63.

194 J. F. Dishinger and R. T. Kennedy, *Anal. Chem.*, 2007, **79**, 947–954.

195 J. F. Dishinger, K. R. Reid and R. T. Kennedy, *Anal. Chem.*, 2009, **81**, 3119–3127.

196 L. Schmid, D. A. Weitz and T. Franke, *Lab Chip*, 2014, **14**, 3710–3718.

197 C. Wyatt Shields Iv, C. D. Reyes and G. P. Lopez, *Lab Chip*, 2015, **15**, 1230–1249.

198 J. A. Vizcaíno, E. W. Deutsch, R. Wang, A. Csordas, F. Reisinger, D. Ríos, J. A. Dianes, Z. Sun, T. Farrah, N. Bandeira, P.-A. Binz, I. Xenarios, M. Eisenacher, G. Mayer, L. Gatto, A. Campos, R. J. Chalkley, H.-J. Kraus, J. P. Albar, S. Martinez-Bartolomé, R. Apweiler, G. S. Omenn, L. Martens, A. R. Jones and H. Hermjakob, *Nat. Biotechnol.*, 2014, **32**, 223–226.