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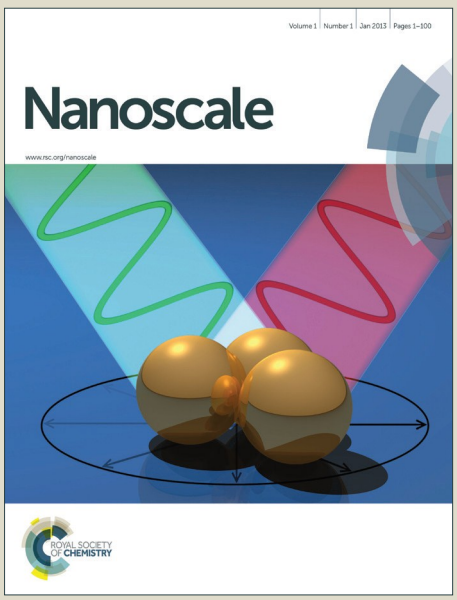

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ARTICLE

Nanoscale Bio-Platforms for Living Cell Interrogation: Current Status and Future Perspectives

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The living cell is a complex entity that dynamically responds to both intracellular and extracellular environments. Extensive efforts have been devoted to the understanding intracellular functions orchestrated with mRNAs and proteins in investigation of the fate of a single-cell, including proliferation, apoptosis, motility, differentiation and mutations. The rapid development of modern cellular analysis techniques (e.g. PCR, western blotting, immunochemistry, etc.) offers new opportunities in quantitative analysis of RNA/protein expression up to a single cell level. The recent entries of nanoscale platforms that include kinds of methodologies with high spatial and temporal resolution have been widely employed to probe the living cells. In this tutorial review paper, we give insight into background introduction and technical innovation of currently reported nanoscale platforms for living cell interrogation. These highlighted technologies are documented in details within four categories, including nano-biosensors for label-free detection of living cells, nanodevices for living cell probing by intracellular marker delivery, high-throughput platforms towards clinical current, and the progress of microscopic imaging platforms for cell/ tissue tracking *in vitro* and *in vivo*. Perspectives for system improvement were also discussed to solve the limitations remains in current techniques, for the purpose of clinical use in future.

1. Introduction

Living cells constantly alter their phenotypes and create specific functions in response to both intracellular and extracellular environments[1, 2]. Equipped with identical genetic codes in the nucleus, cells feature unique genetic performance which orchestrates the mRNAs and proteins to be adaptive to the environmental factors. Cell functions usually involve the expression levels of mRNAs and interactions between proteins, understanding of which eventually help us investigate the fate of a single-cell, such as proliferation, apoptosis, motility, differentiation, mutation, metastasis, etc[3]. On the other hand, cellular phenotype is greatly influenced by extracellular matrix. Living cells keep secreting extracellular vesicles for cell-cell communication or self-defence. Particularly, in stem cell cultures, these autocrine and paracrine cues affect self-renewal, growth and differentiation, and contribute to the initiation of tumours, cellular heterogeneities[4].

Modern techniques those aid in intracellular / external probing have demonstrated their benefits in understanding cell biology and medicines. Living-cell detection tools are invented primarily to disentangle the signalling pathway, characterize the relationships between cells state, environmental stimuli and cell response[5]. Controlling the delivery of stimuli, rapid response read-out, while maintaining the cell alive, are some important factors to be considered in interrogation tools. Currently, polymerase chain reaction (PCR), DNA microarray, western blotting, enzyme-linked immunosorbent assays (ELISAs) are commonly adopted in practical[6-8]. These methods offer high resolution on quantitative measurement, including the exact copy number of target DNA, RNA in single-cell. However, cytolysis is also required, thus losing great significance as the cells of interest are still desired for further investigation[9].

With development of the microscope, trials on living cell observation have been performed for long term. Particularly, recent years have witnessed the rapid growing number of reports on the design of nanoscale bio-chips for living cell interrogation. These systems offer special opportunities to address current technical challenges. While microfluidic devices have shown the single-cell determination by confining the cells and their environments on the chip, nanoscale devices provide the capability of single-molecular manipulation. Such high resolution make possible to tease apart the complex the interaction involved in cellular signalling pathways.

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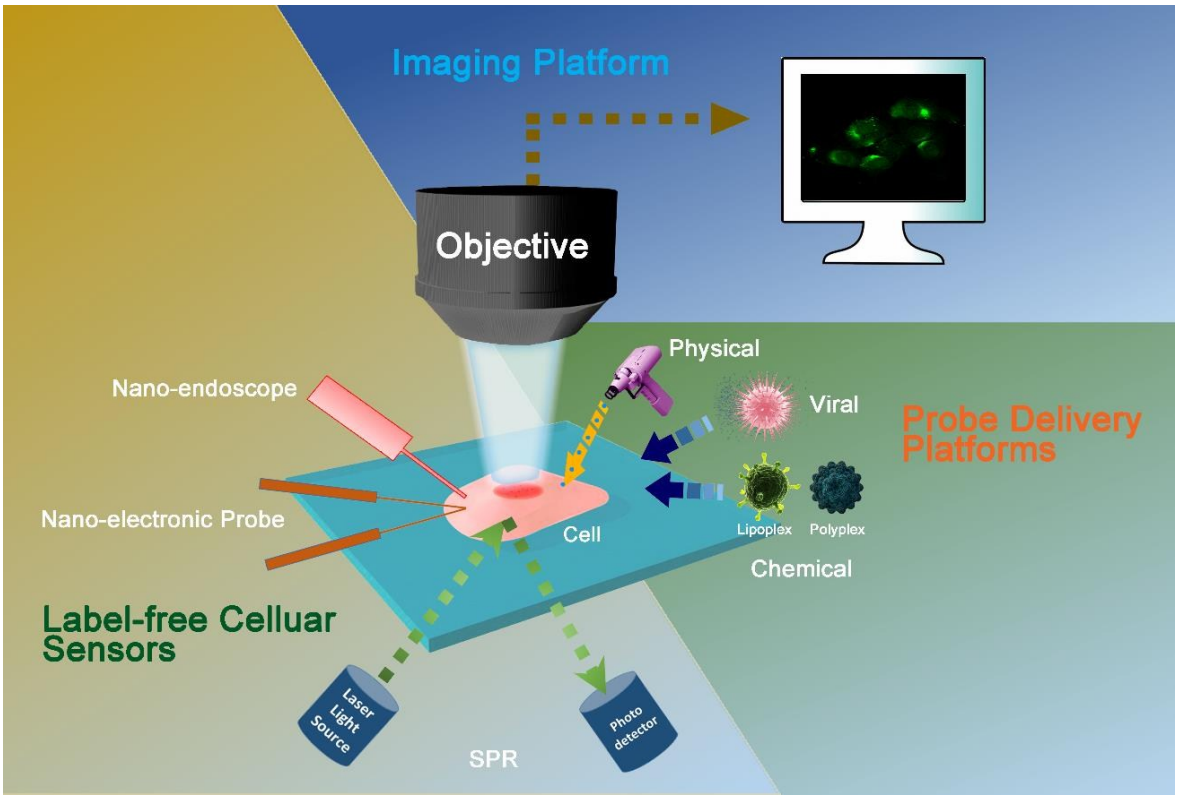

Scheme 1 Single living-cell interrogation on three representative platforms, including label-free biosensors (Section 2), probe delivery platforms (Section 3). Advanced platforms for mass cell analysis are highlighted in high-throughput systems (Section 4) and imaging platforms (Section 5).

In this review, we summarize the state-of-art of nanoscale biochip for probing the living cells. We focus on the typical work related to background introduction and technical innovation of the platforms during the past 10 years. Related techniques for living-cell interrogation are classified into four sections (Scheme 1). We first give insight into the nano-biosensors for label-free detection of living cells. The nanosensors, dependent on fundamentals (i.e., plasmonics, electrochemical, impedance), are discussed in details in terms of the detection objectives (e.g. the binding kinetics of membrane proteins, action potential, endoscope). We then provide an introduction to nanoscale devices for intracellular delivery for probing into living cells. Novel platforms based on viral, chemical and physical methods are presented. Some unique and insightful nano-devices those demonstrated significant technical improvement are highlighted. We next highlighted representative high-throughput platforms for massive cell analysis, providing statistical meaningful data and moving towards clinical application. As the functions of most systems for cellular tracking are assisted with imaging techniques, we then glance over the current progress of microscopic imaging platforms, in vitro and in vivo. Some typical issues remaining in these three prototypes are also included, while prospects are briefly given for the clinical purposes.

2. Label-free Biosensors

Conventional approaches for intracellular detection, such as immunochemistry, ELISAs, microarrays, are implemented by fluorescent labeling consisting of conventional fluorescent staining and the molecular tagging via genes of fluorescent proteins (FPs)[10-12]. However, the major concerns of these techniques include necessity of cytolysis, laborious processing, specificity, and the interference of labels to the protein functions[13]. To monitor the living cells without interrupting normal physiology, label-free platforms seem to be the ideal way for in vivo and in situ detection in primary cells, tissue, or living systems. Indeed, the recent emergency of nanotechnologies have opened up opportunities to involve the nanosensors in living cells detection with high-resolution.

2.1.Surface plasmonic resonance (SPR) nanoprobes

Cell membrane is constituted by lipid bilayer and membrane proteins those attached and embedded in the membrane. Membrane proteins, such as membrane receptors, transport proteins and membrane enzymes, could perform a variety of functions for many fundamental cellular processes, like cell

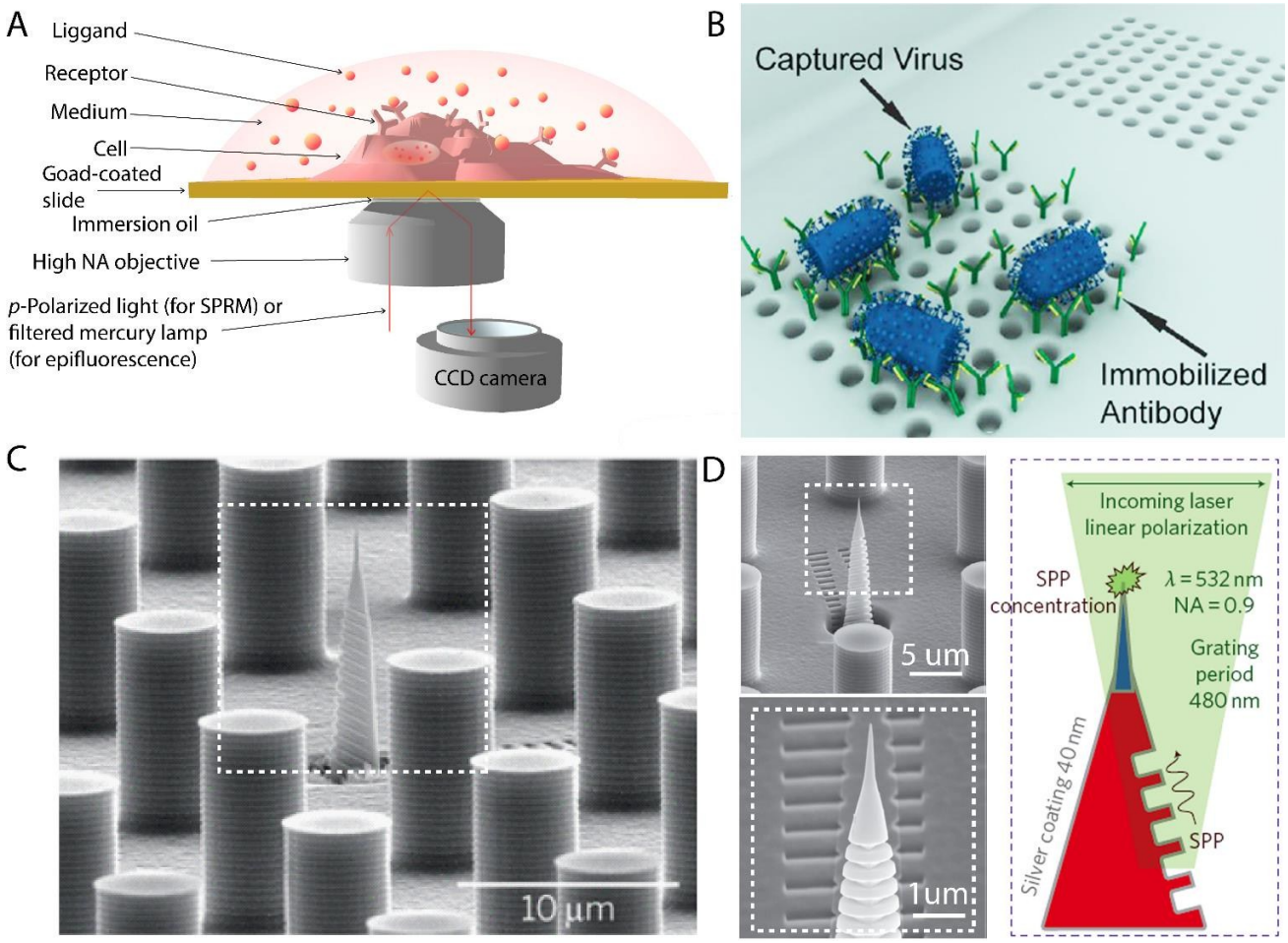

Fig.1 Surface plasmonic sensing and detection. (A) Schematic illustration of surface plasmon resonance microscopy and experimental set-up. (Ref. 24, Nature Publishing Group) (B) Detection of vesicular stomatitis virus (VSV). (C) Super-hydrophobic device with embedded plasmonic nanostructure. (D) Details of the device and measurement principle. Figure B and figure panel (C and D) are reproduced from Ref. 32 and Ref. 34 with permission from American Chemical Society and Nature Publishing Group individually.

signaling, communications and transportation of materials in and out of cells[14]. One of the most significant characteristics of membrane proteins is that they are distributed at varied locations over or embedded in the cell membrane and control varied cellular behaviors dynamically[15, 16]. An analytical tool with high resolution and real-time monitoring of proteins' activities is required to comprehensively understand membrane proteins. Traditional biological techniques such as Western Blot, ELISA and Electrophoresis[17-19] used for measuring binding kinetics of membrane proteins are 'one-time shot', not helpful for real-time monitoring. Although electrochemical biosensors can be employed for continuously monitoring, labels are required to detect non redox-active analytes[20, 21]. Atomic force microscopy (AFM) may give high resolution when measuring membrane proteins[22]. However, AFM is still not very helpful for detecting binding kinetics of membrane proteins due to the contact of the AFM tip to the cell membrane[23]. Surface plasmon resonance (SPR) biosensors can resolve these restrictions, by offering highly spatial-resolution. It can be regarded as a promising tool for studying binding kinetics.

A novel application of plasmonic detection method is real-time monitoring membrane proteins and simultaneously measuring binding kinetics of membrane proteins in single living cells[24]. The analytical tool is surface plasmon resonance microscopy (SPRM)[25, 26] (Fig. 1A), which can provide both optical and fluorescence imaging for the same sample simultaneously. Binding Kinetics of membrane proteins was studied by testing the cultured SH-EP1 human epithelial cells. When prepared wheat-germ agglutinin (WGA) solution flow over the SH-EP1 cells, the WGA would interact and associate with the glycoproteins on the single cells, giving an increase signal in the local SPR sites. The quantitative information about the forward binding and backward denaturing kinetics of the bingding between WGA and glycoproteins in a single cell is generated by plotting the average of SPR intensity versus time resulted in a SPR sensor gram. Besides the advantages of label-

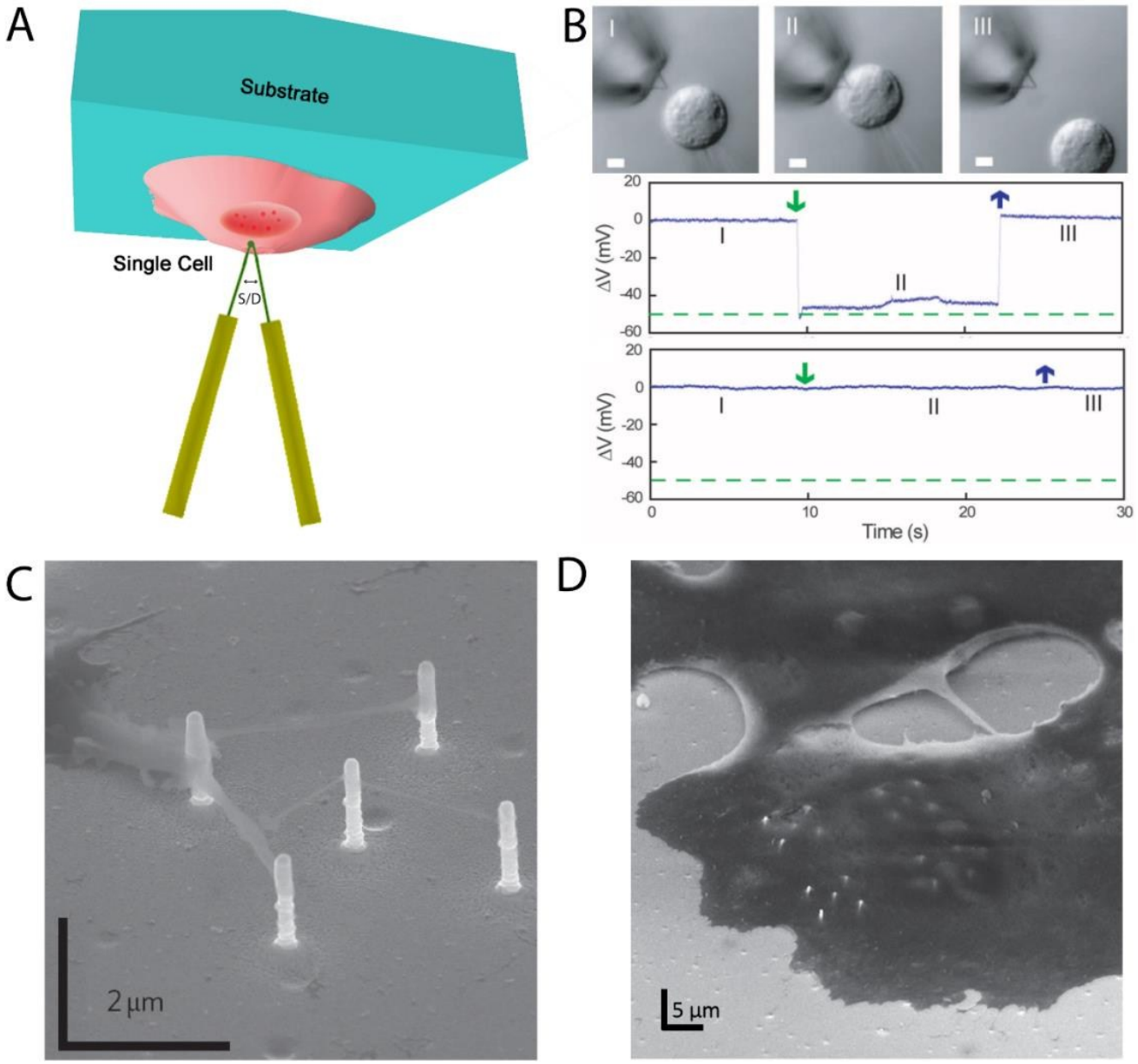

Fig. 2 Nano-electronic sensing and detection. (A) Schematic of a kinked nanowire FET probe. Green regions are nanowire S/D. (Ref. 38, AAAS) (B) Differential interference contrast (DIC) microscopy images (upper figures) and electrical recording (lower figures) of an HL-1 cell and kinked nanowire probe. (C) SEM image of nanopillar electrodes. (D) SEM image of electrode arrays penetrating an HL-cell. Figure B and figure panel (C and D) are reproduced from Ref. 39 and Ref. 40 with permission from PNAS and Nature Publishing Group separately.

free and non-invasive characteristics, moreover, it gives the real-time response of glycoproteins to cellular membranes.

SP-based optical sensors are also particularly promising diagnostic tools for early detection of infectious viral diseases compared with other electrical and mechanical label-free biosensors[27-31]. A novel plasmonic detection method that combines plasmonic sensing and nanofluidics in the same platform enables both the resonant transmission of light and the active transport of fluidics through the periodic array of nanoholes[32] (Fig. 1B). The plasmonic resonance shifting occurs due to the increase of effective refractive index of medium caused by accumulated biomass on the modified functional sensing surface.

The holey array surface is immobilized by antibodies specifically capturing varied kinds of viruses (VSV, PT-Ebola, and Vaccinia). The target virus with varied concentrations flowed through nanochannels to the holey array surface and is captured by the antibodies. The SPP velocity would be decreased according to the accumulation of biomass on the sensing surface. The pronounced resonance shift

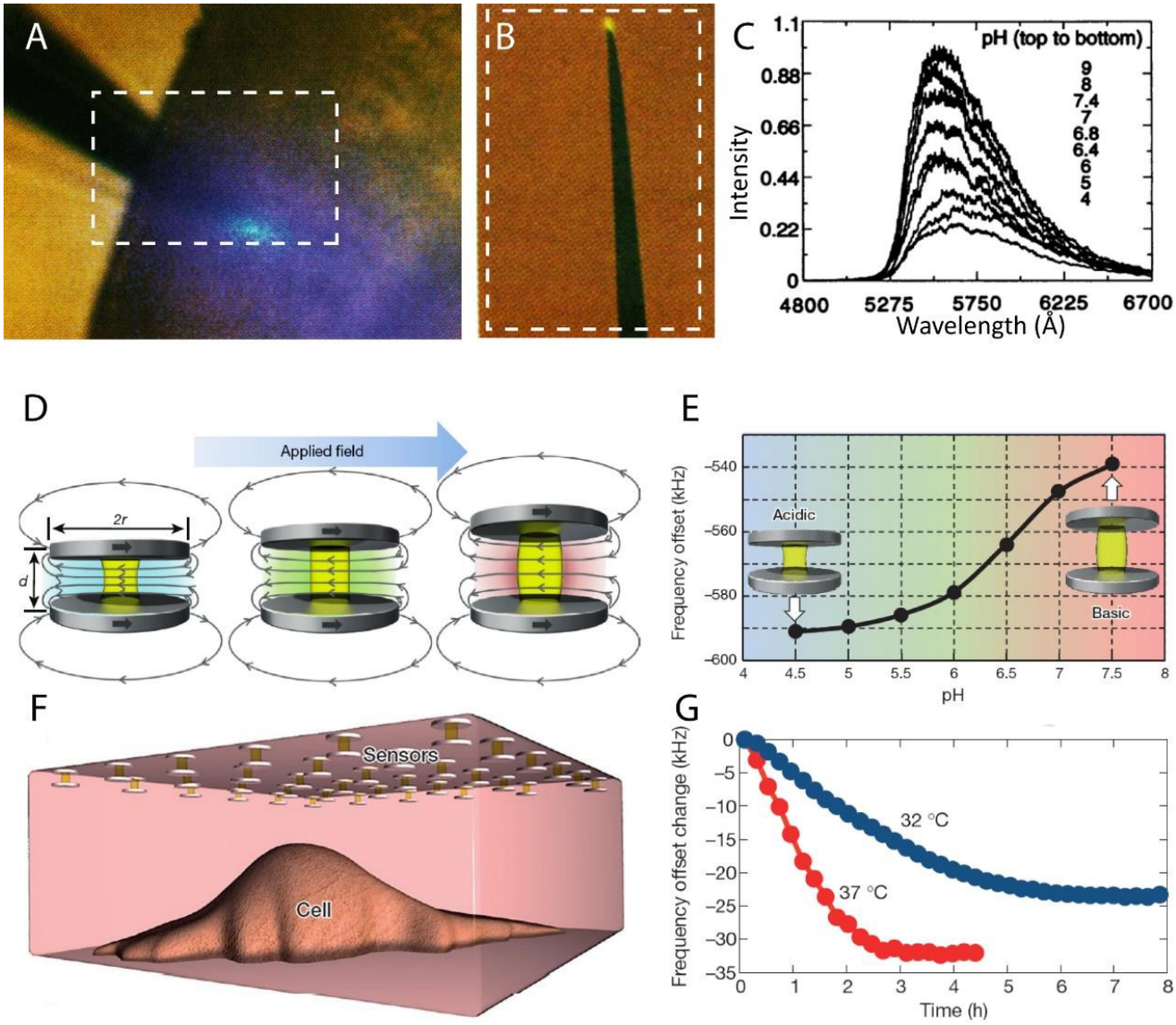

Fig.3 Label-free technology for intracellular pH measurement. (A) Nanofiber sensor is inserted into a rat embryo. (B) Image of the nano-optical fiber pH sensor. (C) Fluorescence emission spectra of optical fiber sensor. (D) Schematic of shape-changing radio-frequency (RF) colorimetric sensors. (E) pH-dependent NMR shifts for nickel-based sensors. (F) Schematic of sensors suspended above MDCK cells in sealed volume of cell growth medium. (G) Shifts in sensor resonance frequencies over time as acidity of cell surroundings. Figure panel (A, B and C) and figure panel (D, E, F and G) are reproduced from Ref. 41 and Ref. 51 with permission from PNAS and Nature Publishing Group separately.

demonstrates this optofluidic biosensor is promising for specific viral-detection, and also it can be possibly expanded into a multiplexed format[32].

Although plasmonic nanosensors are promising devices in the features of high sensitivity, label-free detection and miniaturization, they only can be used for detecting the concentration of analytes greater than ~5 to 20 femtomolar[33]. When the concentration of analytes is at femto- or attomolar levels, it is impossible to drive those limited amount of molecules toward sensing area. The challenge is to overcome this diffusion limit. A new approach (Fig. 1C) shows a super-hydrophobic surface used to overcome the kinetic barrier and localize molecules in highly diluted solutions at the center of plasmonic tip[34], which consists of a silver nanocone with a lateral grating on one side at the center of the array. The surface plasmon polaritons (SPP) was converted by the grating in the silver nanocone. The SPPs accumulates in the nanocone providing an electric-field enhancement (Fig. 1D). At the meantime, the super-hydrophobicity drives the analytes to the tip. At the end of evaporation process, the analytes will accumulate on the silver nanocone, giving a very low detection limit. The analyte, lysozyme, was deposited on the sensing surface at a concentration of 1 fM, ~100 molecules in a 160 µL droplet.

2.2.Nano-electronic probes

Action potentials play an important role in cellular process, the accurate electrical measurements inside single cells or neural tissue has several significant implications in biomedical area. The dynamic ion flow inside cells can generate field potential, which can be detected by field effect transistors (FET) devices[35-37]. NanoFET should have better sensitivity due to the sensitivity of FET is inversely proportional to the dimension of the active device. Moreover, when interfacing with cells, the small sizes of NanoFET can diminish the interfacial impedance and biochemical invasiveness to cells. Intracellular electrical recording is more important than extracellular recording, because it truly measures transmembrane potential of the cells, and it is also possible to detect subthreshold events such as potentials related to synaptic interactions. More importantly, the intracellular recording clearly measure electrical signal within cytosol rather than the membrane signals which represent an average over cells. Currently, the main challenge to realize intracellular recording is related to the geometry of the FET-based probes. To address this issue, a novel approach by using kinked nanowires is proposed by Lieber *et al.*(Fig. 2A)[38]. The voltage sensitive active channel is encoded to the tip of the kink, while the two heavily doped nanowire arms of the kink serve as S/D electrodes. In upper panel of Fig. 2B, the phospholipid-modified kinked nanowire probe was brought close to contract and then moved away from an isolated HL-1 cell (a mouse cardiac muscle cell line)[39]. The measurement of potential versus time clearly shows a sudden potential decease after the cell contacting to the nanoprobe tip, while the unmodified probes exhibited only baseline fluctuations (Fig. 2B). The unique characteristic of the nanoFET probes from conventional intracellular probes is that they can reenter and be retracted from the same position on the same cell for multiple-time measurement without affection. This feature provides nanoFET probes for multiple-time, long-term recording. Fig. 2C shows another kind of vertical nanopillar electrode[40], with the advantage of tight junctions with cell membranes, which could feedback an accurate action potential recording decreasing the impedance between cell-electrode interface and increasing the efficiency of signal collection. Both extracellular and intracellular action potentials of cultured HL-1 cardiomyocytes can be read out in short or long period of time with remarkable signal strength and quality. Scanning electron microscopy (SEM) shows the cell penetrated by the nanopillar electrodes (Fig. 2D). Moreover, the nanopillar electrode is demonstrated that it can alternatively record extracelluar and intracellular action potentials by nanoscale electroporation and resealing processes. Interestingly, the vertical nanopillar electrodes enable the simultaneous recording of drug application. The changes of action potential before and after adding nifedipine (a Ca^{2+} channel blocker), or tetraethylammonium (a K^{+} channel blocker) with different concentrations to the culture medium, can be precisely detected.

2.3.Novel Magnetic resonance imaging (MRI)-based probes

Intracellular pH plays a key role during the testing of living cell and organ functions. At the end of 20th century, intracellular pH had been widely measured via label-free nano-optical fibers since label-free technology realized pH measurement in a single rat embryo (Fig. 3A)[41]. For example, Fig. 3B shows the photograph of 105 μm optical fiber pH sensor, and the related fluorescence spectra for excitation wavelength are also shown in Fig. 3C.

Fluorescent labeling sensors and plasmonic sensors have revolutionary progress in molecular biology. However, optical labels can only probe the near surface areas. The increasing of depth in cell results in the decreasing of the optical resolution and sensitivity. Magnetic resonance imaging (MRI) [42, 43] has been commonly employed in medical diagnostics and clinical research although it is limited in a few aspects due to the lack of sensitivity and multiplexing capability, compared to colored fluorescent labels and plasmonic labels[44-46]. MRI cell tracking is due to the magnetically dephased signal which is generated from the water surrounding cells labelled with contrast agents. The key issue for making multiplexing, high sensitivity radio frequency (RF) sensors is to generate multispectral agents with shaped magnetizable structures to shift NMR frequencies[47-50]. The reason is that different local magnetic fields are dominated by different structure shapes, which can further transform contrast agents into 'colored' RF tags to be used for multiplexed labelling. Very recently, a novel geometrically encoded magnetic (GEM) sensor design based on multispectral MRI agents which include spaced magnetizable disk pairs automatically aligning themselves with applied magnetic fields because of their magnetic shape anisotropy (Fig. 3D) was demonstrated its application in localized PH measurement, ion concentration gradient measurement, simultaneous tracking of cell metabolism and co-localized sensing[51]. In particular, localized PH sensing can assist with the diagnosis of various pathologies of inflammation, ischemia and cancer. PH measurement is based on the changing of disk spacing between nickel and iron sensors which will affect the internal field magnitude and NMR frequency shift (Fig. 3E). When in relatively acidic conditions, protonation will shrink the PH-sensitive hydrogel spacers, decreasing the distance between nickel and iron disks and increasing the magnitude of NMR frequency shift, *vice versa*. Local ion concentrations can be measured based on the gradient of electrostatic shielding by recording the diffusive mixture of phosphate buffer and adjacent water-based agarose gel. The extended operation of GEM sensors in biological fluids was verified by tracking the metabolic rates of Madin-Darby canine kidney (MDCK) cells[51] (Fig. 3F). In a sealed environment, the metabolic production of CO_2 and cell death in the increasingly hypoxic environment would affect cell growth medium's acidification which can be monitored by the GEM sensors (Fig. 3G)[51]. The sensors also can be further designed by biocompatible materials, offering potential responsive NMR spectral shifts. These shape-changing systems have the potential to detect varied environmental and physiological indicators, providing generalizable, MRI-compatible label-free sensors for usage in chemical, biological, medical and clinical research.

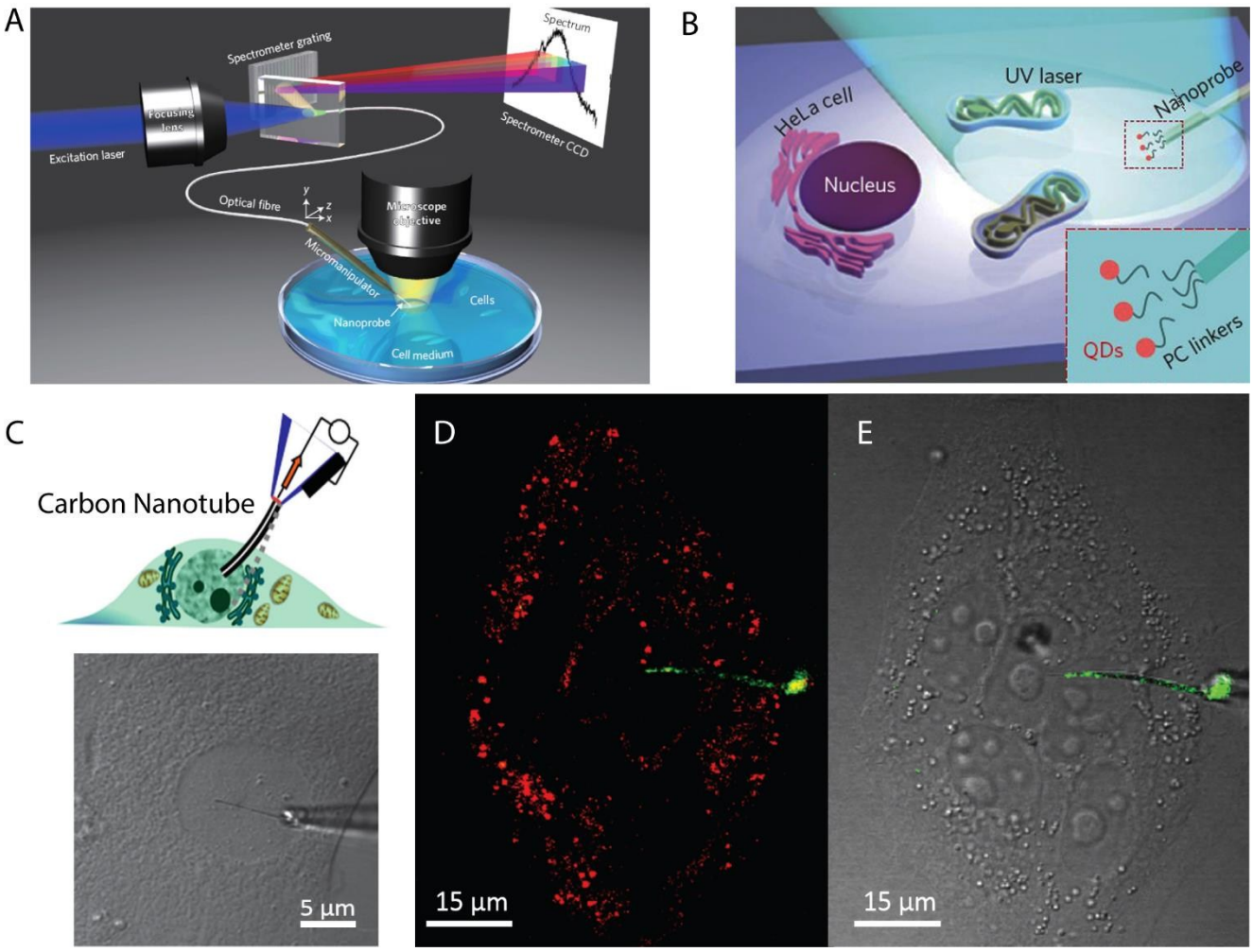

Fig. 4 Single cell nano-endoscopy. (A) Schematic diagram of the nanowire-based cell endoscope system. (B) Schematic drawing of delivery of quantum dots (QDs) by using nanowire-based endoscope into a living cell. (C) Schematic (upper) and real system (rat hepatocyte nucleus, lower) of the carbon nanotube endoscope. (D&E) Fluids and particles flow through the nanotube endoscope. Figure panel (A and B) and figure panel (C, D and E) are reproduced from Ref. 61 and Ref. 57 with permission from Nature Publishing Group separately.

2.4.Single-cell nanoendoscopy

Recently, single-cell interrogation at the nanoscale can be realized on several kinds of nanoprobes, such as pillars[52], wires[53-55] and tubes[56-58], for gentle insertion or penetration into single cell's interior. All those methods allow to explore intracellular action potential, internal drug delivery, electrophysiology[52, 57, 59] and internal endoscopy[57, 60, 61]. However, optical signal transportation across the cellular membrane at the subwavelength level still cannot be realized on such probes based on nanowires and nanotubes.

A novel nanowire-based endoscope system was designed by Yang's group for detecting optical communications in individual cells and the cargo delivery into intracellular sites with minimized affection to the living cell[61] (Fig. 4A). The advantage by using such nanowires is related to its less-invasive manner, which benefits from the ultra-small dimensions and uniform geometry. The payloads could be attached to the nanowire for being delivered into specific sites in the cell. The cargo delivery concept was demonstrated by attaching quantum dots (QDs) to the nanowire tip through photo-cleavable (PC) linkers (Fig. 4B)[61]. After the insertion of functionalized endoscope into the cell, the PC linkers would be chopped by low-power ultraviolet radiation and QDs can be released into specific intracellular sites. Another promising material for making nanoscale endoscopy probe is carbon nanotube (CNTs) due to its electrical conductivity, cylindrical shape, adjustable diameter and mechanical strength[54]. A new kind of CNT-based endoscopy probe consisting of a carbon nanotube connected at the end of a glass pipette tip is shown in Fig. 4C[57]. The nanotubes were filled with super paramagnetic nanoparticles to acquire electromagnetic function that could be employed to provide submicron deflections for precise positioning of the tip inside the cell. The

unique electromagnetic property makes it possible to record intracellular electrical signals and electrophysiology by monitoring cellular Ca^{2+} homeostasis. This carbon nanotube-based endoscope exhibits multifunctional capabilities including fluids transferring, simultaneous fluorescence, optical and electrochemical probing (Fig. 4D& E) [57].

In this section, we have described representative nanoscale biosensors for label-free living cell detection. These devices, rely on four prototypes (i.e. SPR sensors, nano-electronic probes, MRI nano-probes and nanoendoscopy), show impressive advantages over conventional counpterparts, including real-time monitoring, ultrahigh sensitivity, negligible membrane damage and good repeatability. Considering the pratical use in medical fields, however; it is worth to mention that these devices are mainly focused on the innovation of research and design (R&D), but limited to the throughput. To be clinical valuable, advanced systems with capability of high-throughput while maintaning single-cell sensitivity are still highly desirable.

3. Intracellular interrogation on probe delivery platform

The recent progress of label-free biosensors for detection of living cells is discussed in the above section. Implementation of biosensor systems is usually driven by the innovation of sensing approaches (e.g. impedance, current, plasmonics) and nanomaterials (e.g. graphene, carbon nanotubes, nanofibers), which generally offer advantages including high sensitivity, fast response, and high resolution. However, most label-free biosensors are limited to the accessibility of intracellular interrogation. The precise localization and high sensitivity at single-molecular level still cannot be perfectly achieved[62, 63].

In general, efficient intracellular interrogation processes involve functional probes which could be delivered into the

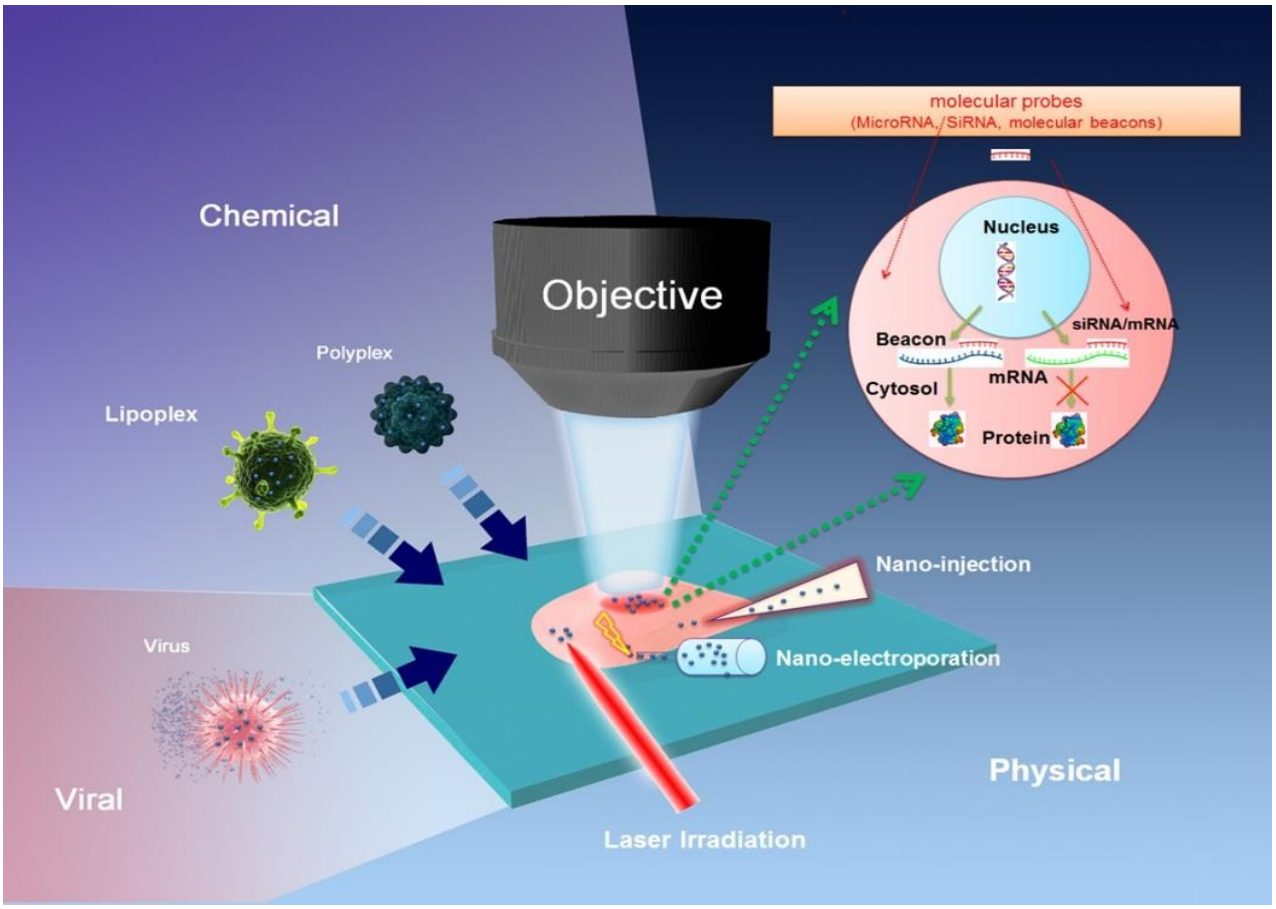

Fig. 5 Advanced nanoscale platforms for intracellular delivery based living cell detection. These platforms are based on three approaches: viral, chemical and physical. Virus / viral vectors loaded with the probes can efficiently and actively transport across the cell membrane and precisely load the probes in both nucleus and cytoplasm (Ref. 69, PNAS; Ref. 70, Nature Publishing Group); Chemical particles (e.g. lipoplex and polyplex), encapsulated with probe molecules, mainly reach to the intracellular destination via endocytosis, the efficiency of which is highly dependent on the interaction between the particles and cell membrane (Ref. 71 & 72, Nature Publishing Group). Physical methods, such as, laser irradiation, nanoelectroporation and nanoinjection, can actively deliver the probes into living cell via cell permeabilization induced by laser-thermal effect, electric field, and mechanical break-down, respectively (Ref. 73, Elsevier; Ref. 74, Nature Publishing Group). For interrogating living cells, molecular probes commonly used include molecular beacons, siRNAs and micro-RNAs, most of which are labelled with fluorescence for indicating the intracellular signals.

cells for the purpose of binding with the targets (i.e. DNA, mRNA, siRNA, proteins) while expressing specific signals (usually fluorescence) that can be read out by extracellular platforms (i.e. fluorescence microscope). Intracellular probes, such as molecular beacons[9, 64], fluorescence labelled - siRNA[65, 66], miRNA[67], oligonuleotides (ODNs)[68], have been studied for long term and now widely established by bio-reagents companies. In this procedure, the key factor is a probe delivery platform with high efficiency, safe to cells, and high-throughput. Herein, we introduce living cell interrogation by delivery of intracellular biomarkers using delivery platforms, including viral-vectors, chemical vectors and physical-based platforms. The prototypes of most systems are designed and developed via the collaboration between engineering researchers (Biomedical, Electrical, Chemical, Mechanical, Materials, etc.) and medical doctors for drug delivery. The major purposes include gene therapy, reprogramming, wound healing and tumor inhibition, all of which is expected to significant regulation of the cellular behaviors in post-transfectional period. In this section, our focus point is on living cell interrogation, including discrimination of cellular heterogeneities, identification of signaling pathway, gene function, protein expression and cancer cell detection, which have negligible or minimal influence on the cellular nature.

While the designed probes have been well-established and demonstrated high reliability, the efficacy of intracellular detection is affected by the delivery platform. In this regard, currently available techniques those could be used for probe delivery are generally classified into three categories: (1) viral-methods; (2) chemical methods; (3) physical methods. Among them, viral methods have been recognized as the most efficient approach for cell transfection. However, concern also raises that virus or viral vectors (retroviruses, lentiviruses, adenoviruses and adeno-associated viruses (AAVs)) typically integrate into the human genome and remain many side effects of immune responses and long-term uncertainty[69, 70]. Alternatively, the chemical method seems to be a promising method for living cell interrogation. Under physiological condition, the cell membrane usually presents negative charge due to the phospholipid bilayer structure composed of both negatively charged phosphatidylserine and phosphatidylinositol on the cytosolic face. To overcome the membrane barrier during the delivery process, chemical methods adopts neutralized or positive charged nucleotide-complex carriers loaded with the markers (with siRNA, miRNA, molecular beacon) to bind with the cell membrane and transport via endocytosis[71, 72]. For physical methods, external forces (e.g. mechanical, electric, magnetic, photonic, etc.) are localized on the cell in order to realize membrane breakdown, followed by direct manipulation of naked probes across the membrane[73, 74]. Compared to the label-free sensors, some modern physical methods have demonstrated to precisely deliver cargo to intracellular destination, with nanoscale resolution. In this section, we will review the advanced platforms for probe delivery, which is benefited with the progress of micro-/nano-technologies. Fig. 5 illustrates some representative nanoscale platforms based on these three methods, which will be discussed in details later. The term of "intracellular interrogation" is this section, refers to the procedure in which the molecular probes are delivered into the living cells for binding with specific targets while releasing the signals which could be read out by the fluorescence microscope. For example, molecular beacon is usually designed to seek for the mRNA in the cytoplasm. In the individual cell which has highly expressed mRNA (i.e. positive expression of the corresponding gene in nucleus), the reporter will show up the fluorescence signal by binding with the mRNA, indicating the high expression of the gene. Different from the function of siRNA and microRNA, molecular beacons are usually designed not to downregulate the mRNA while probing. In some cases, downregulation of mRNA with siRNA and microRNA has great significance to study the behavior of the living cells, such as the role of the endo-gene in drug resistance of the tumor cells, heterogeneities of primary cells, etc.

3.1. Viral vectors for Probing living cells

Viral methods have been recognized as the most powerful tools for cell transfection, with > 90 % efficiency. So far, ~ 70 % reported clinical gene therapy tasks rely on viruses or viral-domains[75, 76]. Particularly, in most cases of cell transfection with functional transcriptional factors in large molecular weight, such as, cell reprogramming (e.g. iPSCs generation with OSKM factors, induced neurons conversion with BAM factors), successes in non-viral methods are rarely reported so far[77-79]. Beside nuclear transfer (cloning) which is single-cell manipulation and fusion with stem cells (electro- or chemical-mediated), viral transduction is considered as the only method accessible to reprogramming with significant efficiency.

However, there are still several limitations associated with the viral vectors for long term need to be seriously considered, including immunogenicity, tropism, limited DNA packaging, and vector production[80]. For instance, in the pioneering work of adoptive immunotherapy, lentivirus was employed to deliver anti-CD 19 chimeric antigen receptors (CAR) for transfection of autologous T-cell, showing high transfection efficiency and long-term persistence of CAR expression[81, 82]. However, the reported chronic lymphocytic leukemia (CLL) patient infused with viral-transfected T-cells presents typical immune responses, including thrombocytopenia, fevers, chills, diaphoresis, myalgias, headache, and fatigue[81]. Severe syndromes were also observed in three acute lymphoid leukemia (ALL) children patients treated with CAR modified T cells delivered with lentiviral vectors[82]. Though successes in anti-tumor efficacy and remission are highlighted, the enhanced toxicity has caused widely spreading discussion on the necessity of safer delivery of CAR. A growing number of non-viral methods have been reported with the efforts to improve the transfection efficiency, persistence of CAR expression, and *in vivo* effect. The ultimate goal is to fully replace viral vectors if the gaps on the performance could be filled[83].

While viral vector encoded with genetic materials (i.e. naked DNA, plasmids, RNAs) are usually employed for gene

therapy, viral-nanoparticles complex have been reported for diagnostics and living cell imaging[69, 84, 85], one important task involved in living cell interrogation. Early cancer detection and real-time monitoring the drug treatment require sensitive imaging techniques for non-invasive detection. Though there are a variety of imaging platforms widely used *in vivo*, such as computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), few of them or modified methods have been empolyed to achieve high sensitivity necessary for identification of early tumor cells or metastasis. Therefore, for imaging sensors, the eventual goal is the capability of specific targeting single-cell or at least in small-scale tissue level. To achieve this, on one side, the discovery and screening of specific ligands towards target cells has provided the high potential to localized imaging in real-time with high sensitivity. On the other side, a variety of nano-platforms, including chemical particles and viral vectors, have also been developed to carry the ligands to binding with cells of interest. Using virus as carriers have been demonstrated with a number of advantages: (i) multiple targets or peptides can be confined in the environments with control over the spacing and ligands; (ii) The self-assembled configuration enables free of morphological polydispersity, which could not be well-established by chemical methods; (iii) The high efficiency of viral-based delivery significantly improves signal-background ratio.

Swiech *et al.* reported an *in vivo* interrogation of gene function in mammalian brain[86]. Adeno-associated viral (AAO) vector was adopted to carry SpCas9 to target Mecp2, Dnmt1, Dnmt3a and Dnmt3b in adult mouse brain *in vivo*. In *in vitro* testing, significant expression of SpCas9 and SpGuide were observed with high co-transduction efficiency of ~80 %, while no adverse effect of viral-transfection was reported. Such a high efficiency allows to comprehensive study of genome function in combination with biochemical, sequencing, electrophysiological and behavioral analysis. The results supported some previous research and indicated valuable conclusions[87-89], including (1) MeCPs gene depletion caused by Cas9 in the dorsal hippocampus could affects contextual learning while showing no significant effect to other cognitive abilities[90, 91]; (2) SpCas9-mediated down-regulation of DNMTs would cause impaired memory formation[92]. In addition, Armbruster *et al.* developed designer receptors which could be activated by a designer drug (DREADD) encoded on retroviral vectors pBabepuro, which could be used to probe whole-brain functional networks and behaviors of specific cell types stimulated and inhibited by chemicals[93, 94]. This method has demonstrated high-resolution, enabling identification of heterogeneities of basal ganglia pathway, selective functional activation of the extended amygdala circuit and many tasks which could not be easily achieved by PETs[95, 96]. Within the recent years, the virus or viral vectors those are still commonly used for *in vivo* imaging include adenovirus / adeno-viral vectors[80, 97-99, 100] lentivirus[101, 102], retrovirus[103-105], and plant viruses, such as cowpea chlorotic mottle virus (CCMV)[106] and cowpea mosaic virus (CPMV)[107-109].

Overall, viral vectors still present as the most efficient vehicles in delivery of fluorescence / functional molecules, especially for *in vivo* living cell imaging and gene identification although it cause wide safety concerns. However, it is still worth to notice that the clinical trials on using other non-viral methods have been growing. For example, Barbosa *et al.* particularly claimed they successfully driven fluorescence proteins (GFPs, RFPs) in neuronal stem cells using electroporation *in vivo*[110]. The labelled cells were repetitively imaged in order to directly observe the conversion of stem cells into post-mitotic neurons. This work may provide valuable information on *in vivo* cellular asymmetric division and regeneration[111].

3.2. Probe Delivery with Chemical Vectors

The basic concept underlying chemical methods-based delivery is that the positively charged chemical functionalities are used to neutralize the negatively charged payload, making the payload/chemical complex slightly positive to be attracted by the negatively charged cell membrane[112]. During the uptake process across the membrane, various endocytosis pathways, including clathrin-dependent, cavelae-dependent, macropinocytosis, and clathrin/caveolin-independent endocytosis, are involved[112]. Based on the properties of the reagents used for neutralization, this method is classified into following four categories: calcium phosphate, dendrimers, cationic polymers, and cationic lipids.

Pioneered by Graham *et al.* in 1973, calcium phosphate based delivery is currently widely used due to its relatively low cost compared to other methods. The mixture of negatively charged phosphate with the positively charged calcium / probe solution results in a fine precipitation of probes containing calcium phosphate, which is then taken up by cells via endocytosis (Fig. 6A)[113]. This method is very useful for some delicate cells uptake, such as neuronal cells, since they are extremely sensitive to the micro-environmental changes caused by probes. However, the major drawback lies in its low efficiency with only 1-5 % on average. Thus, the major trend for calcium phosphate based transfection is to increased its efficiency so as to broaden its application in gene delivery including cell interrogation[114].

Dendrimers utilize highly branched surface functionalities to bind and deliver the payload into living cells (Fig. 6B). It is also employed for living cell interrogation by several different groups[115, 116]. For example, by labeling the tumor vasculature targeted cyclic peptides and blood brain barrier-permeable Angiopep-2 peptides to the polyamidoamine-G5 dendrimer, Yan *et al.* developed a two-order targeted nanoprobes for visualizing brain tumor[117]. Compared to commercially available MR contrast agents, it can precisely delineated the boundary of glioblastoma with high signal / background ratio thus being accessible for real-time tumor visualization during surgery[117].

Cationic polymer based delivery utilizes the linear or branched cationic polymers as the carrier to condense and deliver the payload in the stabilized nanoparticles (Fig. 6C). The commonly used cationic polymers include poly-L-lysine

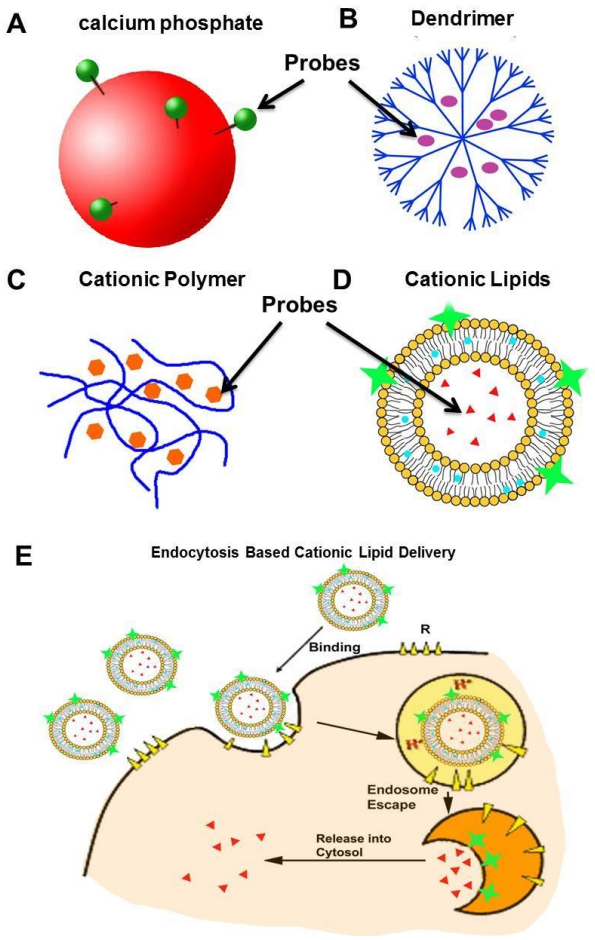

Fig. 6 Chemical cargos preloaded with probes for intracellular delivery via endocytosis. (A) Calcium phosphate is a nanoparticle mixed with negatively charged phosphate and positively charged calcium / probes (Ref. 113, Elsevier, Ref. 114, Nature Publishing Group). (B) Dendrimer is a ranched organic compounds binding the functional probes (Ref. 115 - 117, ACS). (C) Cationic polymer based delivery utilizes linear or branched cationic polymers as the carrier to condense and deliver the payload in the stabilized nanoparticles Ref. 118 and Ref. 120, Elsevier; Ref. 119, Hindawi; Ref. 121, ACS). (D) Cationic lipids encapsulate the functional probes in to its lipid bilayers structures, and release them after deliver into the cells Ref. 119 and Ref. 126, Bentham Science; Ref. 125, Springer; Ref. 127, RSC. (E) Cationic lipids encapsulated probes for intracellular delivery via endocytosis.

(PLL), polyethylenimine (PEI), cyclodextrin, and chitosan[118-120]. As the second polymer introduced for chemcial modifications after PLL, PEI and its derivatives are extensively studied in this scenario. For instance, by encapsulating dozens of quantum dots (QDs) into amphiphilic PEI derivatives (amPEI), Park *et al* showed that the QD-amPEI composite has very efficient QD cellular labeling compared to conventional lipofectamine based delivery[121]. This composite is also very promising for gene delivery, cell-specific labeling, and ratio-metric oxygen sensing, thus providing an ideal platform for living cell interrogation.

Cationic lipid based delivery system includes liposomes, solid lipid nanoparticles, micelles, and micro-emulsion (Fig. 6D). Among them, the lipid nanoparticle based transfection has been recognized as promising delivery system due to its biocompatibility and ease of production. Compared to other methods mentioned above, it usually exhibits higher efficiency in transfection since the positively charged lipids prevent the enzyme degradation and enhance the cell endocytosis[112]. Using this method, our group successfully addressed the biodistribution and cellular trafficking of gene drugs in various models[122, 123]. More recently, we synthesized the transferrin-conjugated lipid nanoparticles (Tf-NPs) for the siRNA delivery in acute myeloid leukemia cells[124]. By encapsulating the Cy5 labelled siRNA into Tf-NPs, they studied the *in vivo* biodistribution by IVIS imaging in NOD-SCID mice. The promising results obtained suggest that the specific receptor-targeting NPs might provide a useful strategy for gene delivery.

Practical advantages of chemical vectors over their physical counterparts include high-throughput capability and easy manipulation, which are friendly to most medical researchers. Current challenge for intracellular probe delivery with chemical vectors is the extra toxicity caused by the artificial carriers[125, 126]. However, efforts towards decreasing the inherent cytotoxicity usually result in the compromise of transfection efficiency. Meanwhile, as the vehicles of chemical particles for cellular delivery are based on endocytosis, the efficiency must depend on the types of cells while the whole procedure is stochastic (Fig. 6E). While various strategies are applied to achieve high efficiency with low cytotoxicity[119, 127], the gap still remains and more work are needed to overcome this paradox.

3.3.Physical-based Nano-platforms for Probe Delivery

Compared to chemical methods, physical methods, by which the cargo is directly transported across the cell membrane, can provide more straightforward and efficient routes for living cell interrogation[128, 129]. There is no requirement on packaging / modification of probes (e.g., siRNA, molecular beacons, fluorescence-labelled ODNs) in order to resist against the endocytosis in physical procedure. Moreover, cell membrane perturbation induced by physical forces could significantly reduce the cellular immune-response to the exotic molecules that is 'sneaking' into the cytosol[73, 130]. Herein, we will briefly introduce the physical-based nano-platforms for active transportation of probes with their latest progress. Classified by the mechanism for membrane break-down, three techniques, i.e., injection, nano-laser platform, and nano-electroporation, are particularly highlighted.

3.3.1. Injection. Injection technique typically adopts a fine pipette to pierce through the cell membrane. Through the established pathway, cargos (probing molecules here) will be pushed to desired locations inside the cell. Due to the dimension of the cell (typical in the range of 1 to 100 μm), the tip of the pipette should be narrowed down to several μm to avoid considerable membrane damage, therefore the early prototypes is also called microinjection. In a fine-controlled experiment, i.e. manipulated by

microscope stage and motor-based arms, it is possible to control the delivery destination, in either cytoplasm or nucleus, while achieving 100 % transfection efficiency. The dose of delivered substances can be precisely controlled. All of these characteristics are superior to other physical counterparts[130].

In early period, microinjection was majorly used for nuclear transfer (clone) in germ cells. There was a critical requirement on the cell size, probably due to the limitation on the tip fabrication and manual manipulation[131, 132]. However, almost all types of cells can be manipulated which is independent from the cell size, which is realized by the progress of the fabrication techniques for tip miniaturization and the enhancement on automation precision. Interestingly, nanoneedles, used as label-free biosensors (as introduced above), have also been widely applied for mediated delivery of probes to study intracellular enzymatic activities, signaling pathways, heterogeneities of gene expression, or immune / electrophysiological activities in response to external stimulation. Park's group reported the use of vertical silicon nanowire array for the delivery of kinds of biomolecules into both cell lines and primary cells (Fig. 7A)[63, 133-135]. This platform demonstrated minimal cell damage and high penetration efficiency (correlated to delivery efficiency). The needles (diameter: ~150 nm, length: 3 μm) have demonstrated as compatible with live-cells for long-term culture, which allows standard microscope to image the phenotypical responses to biological effects. For example, by delivery of Nav1.X-targeting siRNAs, the neurons substantially reduced the sodium current in depolarization steps[63]. In another trial, chronic lymphocytic leukemia (CLL) primary cells were delivered with LEF1 siRNA to study the cellular heterogeneity. The results showed downregulation of LEF1 mRNA by siRNA could significantly influence the survival of CLL cells. As LEF1 is one activator gene to the Wnt signaling pathway, this hinted Wnt signaling might influence the pathogenesis of CLL via LEF1/TCF complex[133]. Interestingly, such nanowires were adopted to electrically interrogate the HEK293 cells, with the unique advantages, including (i) mapping the neuronal network; (ii) simultaneous detection intracellular action potential and synapses[134]. Similar benefits were also performed by Cui group using a silicon nanopillar platform[62, 135, 136]. Additionally, polymer based nanostraw, nanofibers and carbon nanotubes were also claimed to efficient cellular penetration and intracellular investigation[137-139]. Recently, the attempts to *in vivo* application have been reported using biodegradable silicon nanoneedles[140]. However, to reveal reliable and important intracellular information at gene level, biostatistics should be analyzed based on a large number of cells which highly demands high-throughput platforms. Therefore, current nanoinjection techniques still require a relatively long-term to be applied in clinical usages.

3.3.2. Electroporation. Cell membrane, mainly constituted with lipid bilayers, is sensitive to local electric field. When the applied voltage is beyond a specific threshold, called transmembrane potential, the cell membrane will be transiently permeabilized[141]. Trials of using electro-permeabilization have been initiated as early as 1970s, by Neumann and other researchers[142, 143]. Understanding the fundamentals of electroporation can be reached by both biology (the formation of hydrophobic or hydrophilic pores)[144] and physics (models of transmembrane potential)[145, 146]. In practical, bulk electroporation (BEP) systems have been used for decades *in vitro* and *in vivo*, and now become commercial available (see BioRAD, Neon Nucleofector, etc. for details). However, BEP need a couple of bulk electrode to induce the electroporation. Cells / cargo mixture are placed in a dielectric chamber and are shocked with a high voltage (typically, 1000 V or higher). Although with high-throughout, this design usually results in serious damage and low efficiency[147].

To address the concerns on transfection efficiency and safety caused by BEP, micro-fluidics based electroporation (MEP) has been introduced to offer a much safer environment[148, 149]. In MEP system, a single-cell is positioned near a microchannel[150] or a micro-electrode[151], with dimensions similar to the cell size (~ 10 μm). These configurations can intensify the electric field with several orders of magnitudes, achieving single-cell electroporation with a safe voltage, e.g. less than 10 V. Nevertheless, since both BEP and MEP's delivery behaviors is based on diffusion, thus the transfection is totally stochastic with a large variation of doses between cells. So it can be concluded that dosage control plays an important role in discriminating heterogeneities with single-resolution, neither of them are favorable to living cell interrogation. Melosh's group reported a nanostraw-electroporation platform which enables high-throughput cell transfection[152, 153]. The nanostraws were fabricated by atomic layer deposition (ALD) of alumina and reactive ion etch (RIE) on a commercial track-etched polycarbonate (Fig. 7B). Interestingly, the nano-straw can also directly pierce into the grafted cells for intracellular delivery, as nanoinjection introduced above[154]. A low-voltage electric field can significantly increase the efficiency. With advantages of dosage control and sequential transfection, the system could reveal that high dose of the DNA plasmid could lead to the apoptosis.

In 2011，our group reported a nanochannel electroporation (NEP) to deliver the cargo into single-cell with precise dose control[155]. A single-cell is positioned against a single nanochannel, while the to-be-delivered cargo on the other side of the nanochannel, as shown in Fig. 7C. The high resistance of nanochannel enables safe poration under a high voltage (typical 100–200 V) electric field. Transfection uniformity and dosage control are achieved due to the high-speed electrophoresis of surface charged cargo (siRNA, DNA, peptides, plasmids) within the nanochannel and 'shot' into the cell. Deterministic interrogation of single-cell, with negligible damage, has been demonstrated with different types of cells. For instance, Mcl-1 siRNA was injected into K562 cells (myelogenous leukemia) to investigate siRNA induced mRNA down-regulation and the intracellular defense behavior. A clear threshold of siRNA dose for cytolysis can be identified with dose control, which could not be achieved by any other previous electroporation systems. A large plasmid encoded with OSKM transcriptional factors was transfected into mouse embryonic fibroblast cells for cell reprogramming (iPSCs), while the post-transfectional behaviors were studied[156]. 3D NEP systems were recently developed using cleanroom-based nanofabrication (i.e., projection photolithography and deep reactive ion-etch) for high-throughput cell transfection[157, 158]. Dependent on the dimension of the chip and density of nanochannel array, the 3D NEP is able to handle maximum million cells per chip (Fig. 7D). In a case study to interrogate the heterogeneity of chronic lymphocytic leukemia (CLL) stem cells, GATA2 molecular beacons were designed to identify GATA2 mRNA and indicate fluorescence. Sorting out GATA2 ^{high} cells allows us to study the gene function of GATA2 in initiation of CLL diseases *in vivo*. D. Espinosa's group developed a nanofountain probe electroporation (NFP-E) for in situ single-cell transfection[159]. A hollow AFM tip is used as the top electrode or cell electroporation, while the cargo is delivered into the cell through the inner of the tip (Fig. 7E). The system delivered a DNA-based beacon to detect of glyceraldehyde 3-phosphate dehydrogenase and a RNA-based

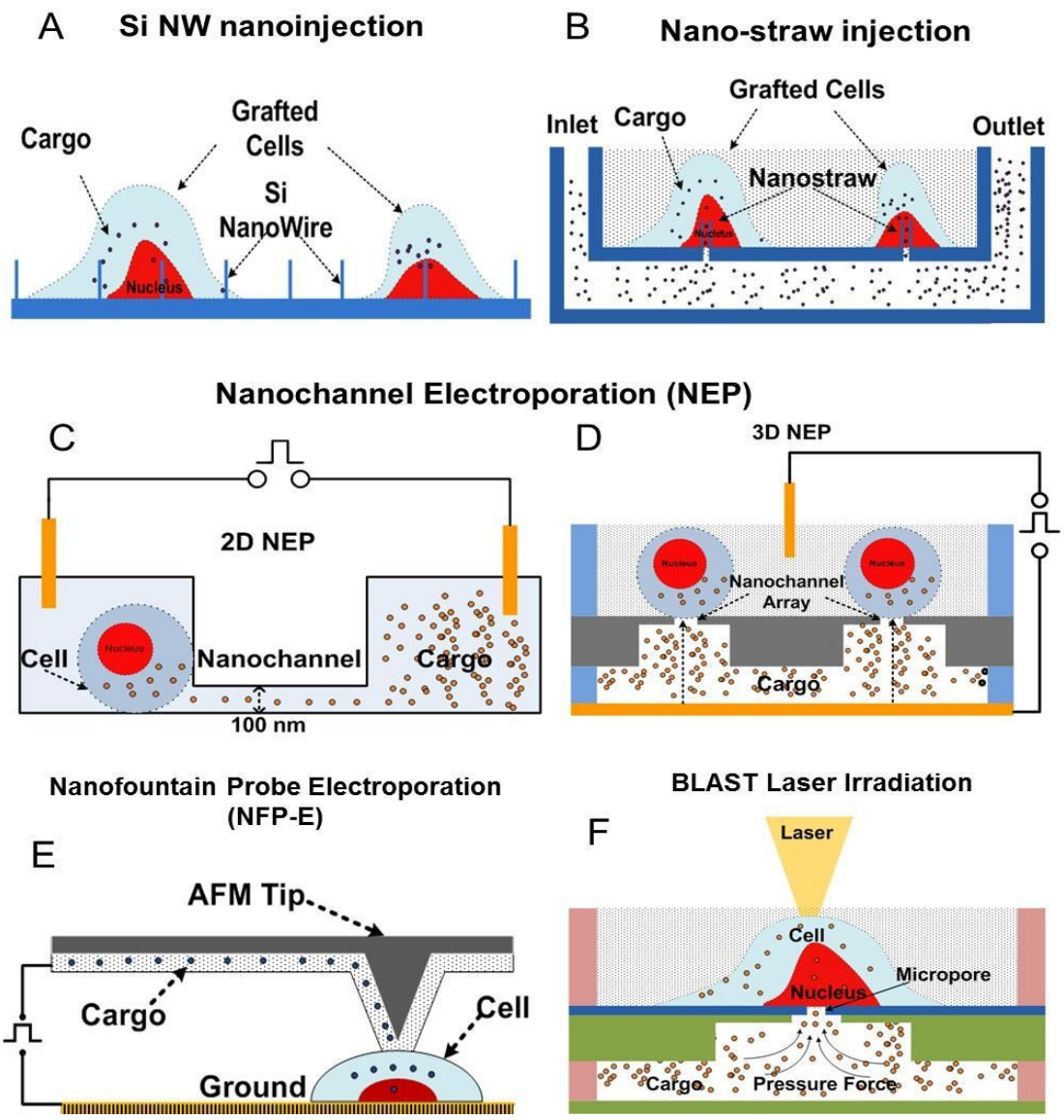

Fig.7 Living cell interrogation can be achieved with advanced nanoscale devices for physical-based intracellular probes delivery. (A) Si nanowires can directly pierce through the cell membrane of the grafted cells, via which the cargo can diffuse into the cytosol for intracellular probing (Ref. 63, PNAS; Ref. 133 &135, ACS; Ref. 134 & 136, Nature Publishing Group). (B) Modified nano-straw can enhance the delivery efficiency by penetrate the cell membrane while establishing a delivery pathway in which molecules can diffuse into the cells Ref. 152 & 154, ACS; Ref. 153, Nature Publishing Group). (C) 2-D Nanochannel electroporation (NEP) demonstrates the capability of precisely control the dose for molecules delivery into living cell (Ref. 155, Nature Publishing Group; Ref. 156, Wiley). (D) 3D nanochannel electroporation (3D NEP) are currently reported to handle million cells on a single-chip, which can meet the clinical and medical requirements (Ref. 157, Wiley; Ref. 158, RSC). (E) Nanofountain probe electroporation (NFP-E) shows flexible movement of a hollow AFM tips and local-electroporation for delivery the cargo into single-cell in-situ (Ref. 159, ACS). (F) Efficient delivery of large cargo (such as bacterium) was demonstrated by laser irradiation platforms combined with 3D nanopores (Ref. 164, Nature Publishing Group).

beacon to measure the sequence cloned in the green fluorescence protein mRNA[160]. Moreover, versatile electroporation platforms were also reported for injecting stimulation effectors while detection of the real-time responses. K. Kitamura *et.al.* patched single-neuron with miniaturized electrode for electroporation mediated-delivery fluorescence dye and plasmids, while recording the stimulated action potentials and transgene expression *in vivo*[161]. Xie and others record intracellular action potential by nanopillar electroporation *in vitro*[62]. Although showing unique advantages from various aspects, it is still worth to mention that several potential issues remain in nano-electroporation systems. First, the delivery efficiency is significantly affected by the cell types, size, morphologies, etc. Second, due to high current density, the process of electroporation may cause irreversible membrane breakdown and cell damage. Furthermore, these techniques show 'smart-designs' which are manufactured with advanced and high-cost fabrication methods. Most of them demonstrated impressive benefit on single-cell manipulation and genetic modification, which still has a long way to be commonly

Table 1 Summary of probe delivery platforms for intracellular detection

Category	Platform	Interfacial Dimension	Materials	Efficiency	Feature	Limitation	Throughput	Reference
Virus	Adenovirus	8kb - 30 kb	dsDNA	High	Extemely efficient Transduction	Capspid mediate a strong cytocoxicity and inflamatory response	High	97 -100
	adeno-viral vectors	<5 kb	ssDNA		Low inflamatory Potential; episomal expression	Small package capacity		80, 97 -100
	lentivirus	8 kb	RNA		Low inflamatory Potential; Integration; Persistent gene expression	Concern about oncogenesis due to integraton		101, 102
	retrovirus	8 kb	RNA					103 - 105
Chemical	Calcium phosphate	10 -100 nm	Ca10(PO4)6 OH2	Low	biocompatible	low efficiency	High	113, 114
	dendrimer		PAMAM poly(propyl enamine)	Medium	biocompatible			115 - 117
	cationic polymers	<1000 nM	PLL, PEI, CD, Chitosan	Medium /High	biocompatible	toxicity		118 - 121
	cationic lipids	100-200 nm	DODMA, DOTMA, DOPE, DMRIE, DDAB	High	high efficiency biocompatible	toxicity		112, 122 - 124
Nano-injection	Si NW Array	~150 nm	Si	Low	1. Direct Injection 2. Low cell damage	1. Depend on adherent cells 2. Long-term cell damage is unkonwn	Low	63, 133 - 136, 140
	Nanopillar Array	150 nm	Platinum		1. Both nanoinjection and electroporation; 2. Used for detection of intracellular action potential	1. Depend on adherent cells 2. Low efficiency in cargo delivery	Low	62, 135, 136
Electro-poration	Nano-straw Platform	~100 nm	Poly-cabonate membrane	High	Low-voltage with high cell safety	1. Diffcult to be dosage control; 2. Hard to deliver large cargo	High	152 - 154
	Nanochannel Electroporation	100-600 nm	PDMS/Si	High	1. Dosage control; 2. minimal cell damage; 3. uniform transfection	Voltage need to be optimized for safe transfection	Single-cell/ high-throughput	155 - 158
	Nanofoutain Probe Electroporation	~800 nm	Si AFM tip	High	In-situ cell transfection	1. Extremely low throughput; 2. impossible to handle suspension cells	Single-cell	159, 160
Laser Irradiation	Laser Chemical	-	800 nm femtosecond laser, facilitated with carbon	High with small molecules	High cell viability	Inactive cargo delivery	Medium	163
	BLAST laser system	<1000 nm pore	Si nanopores with Ti Membrane	High	Enable delivery of large cargo (500 nm)	Active delivery is faciliated with pump	High	164

used in clinical trials and daily medical therapy.

3.3.3. Laser Irradiation. Cell Cell permeability is generally influenced by thermal effect applied onto the cell membrane, which allows a focused-laser beam to create tiny, localized perforation for administration of foreign molecules[73]. The efficiency of the delivery generally depends on (i) the difference of osmotic pressure across the membrane this is determined by the concentration of the target molecules, and (ii) the size of the pores generated on the membrane controlled by the power density, duration and wavelength.

However, considering that openings in cell membrane is difficult to be well controlled and is various in different cell type, laser methods have obvious drawbacks on cell viability. The molecules uptake dependent on osmotic pressure is slow and less efficient[162]. To address these, Chakravarty *et al.* reported a modified laser

irradiation approach for intracellular delivery with improved efficiency[163]. In this system, a femtosecond laser pulse was applied to both create transient opening in the cell membrane and activate the surrounding carbon black nanoparticles, which subsequently generate shock-wave to facilitate the environmental molecules (siRNA, genes, etc.) transported into the cells. Recently, Wu and co-workers report a high-throughput laser platform (BLAST) for delivery of large cargo into mammalian cells with high efficiency (Fig. 7E)[164]. Cells are loaded on a porous SiO_2 membrane, in contact with nanopores coated with metallic layers. A laser is applied to scan over all the cells via nanopores, while the thermal effect generated on the metallic layer will locally perforate the cell membrane. To facilitate this delivery, a fluid pump is used to actively push the surrounding molecules into the seeded cells. The system enables delivery of large cargo, such as 500 nm sized nanoparticles, even bacterium, into living cells. Based on this, it suggested iglC gene plays an important role in intracellular replication and escape of *Francisella*, a facultative intracellular bacterium which could cause tularemia. However, laser irradiation still remains a relatively novel method. Compared to electroporation systems, the high cost and complicated laser system determine the limitation in application of medical research. Therefore, though an increasing number of laser platforms have been reported for *in vitro* proof-of-concept, the necessity, or uniqueness of using this approach for gene delivery has not been evidenced, in observation of the success of electroporation.

Besides the physical methods mentioned above, acoustic is also used to open cell membrane by triggering cavitation bubbles with fluid flows, which significantly increases membrane permeability[165]. Squeezing the cell body through a narrow-microchannel for transient abruption of membrane was also reported for gene delivery[166]. In Table 1, the characteristics of each probe delivery platforms are summarized. Nevertheless, nano-chips employing these approaches for intracellular delivery are less reported in previous. Descriptions on some of these systems have been excellently reviewed previously[73, 130, 167, 168].

4. High-throughput Platforms for Mass Cells Analysis

The above-mentioned nano-devices have demonstrated unique advantages for precisely handling single-cell. However, statistical meaningful analysis is usually dependent on the data collected from a large population of cells. For clinical purpose, particular in the scenarios of genomic analysis and intracellular marker screening, advanced systems for deterministic analysis usually require the capability of high-throughput while maintaining the single-cell resolution. In this section, we highlight several representative platforms for controlled operation of cells in high-throughput way. These techniques, based on the continuous cell-flow mechanism, have demonstrated with high-performance on localizing, lysing, sorting or mixing cells with reagents. The most popular and successful prototype is microfluidic assay systems where cells are suspended and flow with liquid phase driven by external forces [169]. Compared to aforementioned single-cell focused nano-devices, these systems have demonstrated successful translation from research and design (R&D) to clinical use.

4.1. Flow cytometry-based platforms

Flow cytometry (FC) is one of the most effective methods to measure physical and chemical attributes of mass cells with high accuracy at single-cell level. Over the past decades, the technique has been transformed from lab study into the gold method for rapid quantitative analysis and individual cell subsets sorting from a heterogeneous population. The rapid development of opto-electrical and fluorescence labelling technologies accelerates the progress of the modern FC analysis technology. Some multi-laser flow cytometers have been developed for massive parallel sorting or analysing cells. For instance, a multi-channel FC platform, with 18 or more fluorescence channels, has recently been reported to simultaneously obtain multi-parameters of heterogeneous cell populations. [170].

Conventional FC system owns high speed, but is limited on the information contents for individual cells. To address this issue, imaging flow cytometry (IFC) method is developed, which provides not only multi-parameter measurement, but also spatial and morphometric data at single-cell level[171]. Currently, high-resolution imaging flow cytometry (HRIFC) can resolve cell derived particles down to about 400 nm[172], thus paving the road for an extensive applications, including biomarker assay, cancer classification, and identification of novel lineage specific or stem cell markers.

Mass cytometry (MC) is a recently developed new technique that applies stable transition metal isotope-labelled antibodies to simultaneously detect protein from single-cell by using atomic mass spectrometry[173]. It enables the detection of 45 parameters per cell while avoiding the issue of emission spectral overlapping between fluorescent dyes in traditional flow cytometry[174]. With high sensitivity, multi-dimensional and single-cell MCs have important contributions to drug screening and therapeutic programs for multiple indications ranging from infectious disease, cancer, inflammatory conditions and trauma.

4.2. Direct cell handling platforms

4.2.1. Bio-electrosprays and cell electrospinning. Jet-based technologies, such as electrospraying and electrospinning, have been widely used in physical, chemical and life sciences for many years[175, 176]. Recent years have witnessed the involvement of these techniques for advanced direct cell handling approaches, offering impressive advantages for the development of modem biomedicine and tissue engineering.

Jayasinghe developed a unique technique, called Bio-electrosprays (BES), to directly handle living cells by creating micrometric droplets under an electric field of high intensity[177-179] (Fig. 8A and B). Droplet microfluidics is a new area in microfluidics research and has been extensively studied and reported due to its various unique advantages [180-183]. Demello *et al.* discussed a lot of methods for the microfluidic encapsulation, and they proposed it is easier to analyze single cell for encapsulating cells within aqueous droplets surrounded by an immiscible fluid[169]. Jayasinghe and Demello demonstrated the coupling of BES with two types of droplet-based microfluidics for controlling cell numbers within living residues[184] (Fig. 8C and D). The size of droplets and the number of cells can be controlled by varying the flow rates and the relative ratio of the aqueous flow to oil flow, respectively (Fig. 8E and F). BESs contribute versatile platforms by integrating various functional units on the microfluidic systems. Notably, it provides numerous opportunities of on-chip cellular labs for multi-factors measurement with fast speeds and precise single-cell control.

Cell electrospinning (CES), a sister-technology of BES, can exploit electric fields between electrodes to directly draw micro- or nano-fibers and scaffolds with living cells and materials[185]. A superposed core structure containing multiple-cell types from the

inner to the outer core was successfully prepared recently[186] (Fig. 8G). Additionally, Fig. 8H clearly shows the distribution of cells to be uniform throughout the fabricated structure.

BES and CES are regarded as high-throughput technology for bio-analysis and diagnostics, tissue reconstruction, drug discovery and targeted delivery [187]. They offer extensive applied environment, ranging from the basic biological sciences to the most applicable in translational medicine for the clinical sciences.

4.2.2. Aerodynamically assisted bio-jetting. Due to strong force with under a high-density electric field (kV/mm), BES and CES systems may be harmful to the operators [188]. To address this issue, Arumuganathar et al. [189] developed a aerodynamically assisted bio-jetting (AABJ) which is capable of drawing cells into a liquid filament by a pressure differential over an exit orifice [179] (Fig. 8I). It allows direct formation of encapsulations by incorporating liquids from different inlets into the chamber of AABJ device (Fig. 8J). AABJ shows an advantage over both BES and CES due to its driving force of air/liquid pressure. The driving force can be applied indirectly on the media over the exit orifice, thus being harmless when handling living cells. AABJ has been applied for handling a wide range of cells and whole organisms[190]. One step further, the approach has been adopted for animal models for validating the integrated effect of AABJ in practical [191, 192]. Meanwhile, AABJ has been studied for improving existing flow cells in flow cytometry by reducing its present footprint and also removing the need for sheath flow.

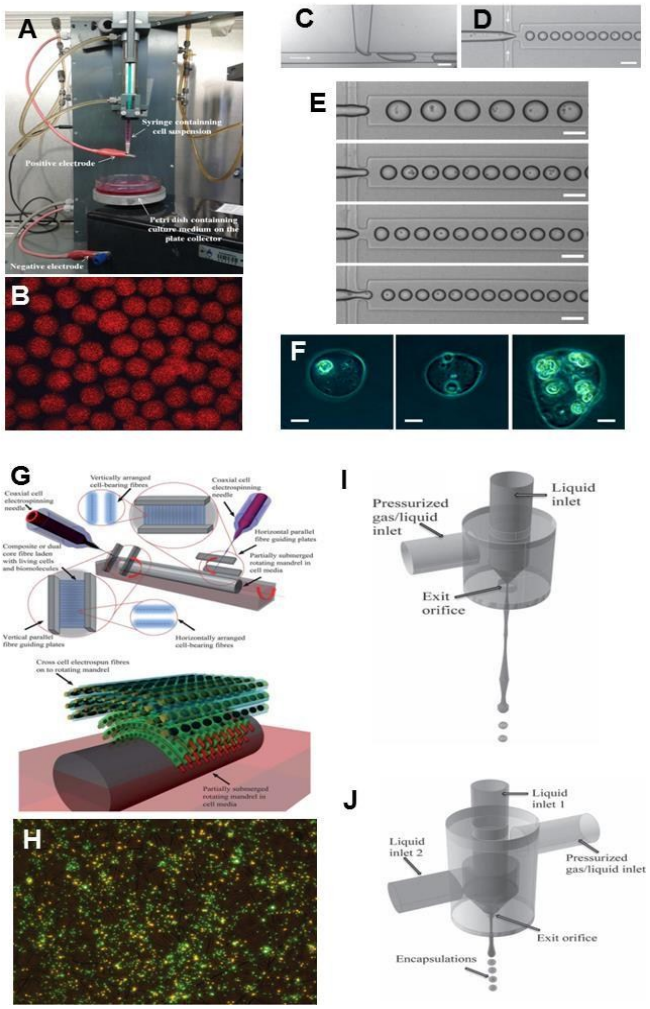

Fig. 8 High-throughput platforms for massive cells manipulation and interrogation. (A) BES equipment for manipulating cells suspension. Reproduced from Ref. 178 with permission from AIP. (B) BES spherical cell-bearing beads (Reproduced from Ref. 179 with permission from Taylor & Francis Group). (C) T-junction microfluidic device for droplets. Scale bar is ~100µm. (D) Flow-focusing microfluidic device for droplets. Scale bar is ~100µm. (E) Droplets containing single and multiple cells at the flow-focusing configuration. Scale bars: 100µm. (F) Cells encapsulated within alginate. Scale bars: 20µm. (C,D, E & F reproduced from Ref.184 with permission from IOP). (G) CES equipment for generating cross stitched living scaffolds (Reprinted from Ref. 186 with permission from RSC). (H) CES scaffold with multiple cell types (Reproduced from Ref. 186 with permission from RSC). (I) Single AABJ device for preparing droplets. (J) Coaxial needle AABJ device for preparing encapsulations (I & J reproduced from Ref. 192 with permission from Wiley).

4.2.3. 3D droplet microfluidic system. Conventional platforms for high-throughput gene screening is based on high-density micro-well plates and robotic dispensing systems [193], which is limited in wide applications due to the high cost of robotic machinery and the extra use of expensive biological reagents. Alternatively, droplet-based microfluidic systems present a promising opportunity for high-throughput biological analysis due to their minimal consumption, low fabrication costs and reduced analysis times [194]. Demello group[195] developed a 3D droplet-based microfluidic device with three PDMS layers, in which concentration gradients spanning 2 orders of magnitude (500 to 5.6 nM with FITC-antibody) can be continuously generated. Rapid enzymatic activity assays and drug cytotoxicity assays on bacteria successfully confirmed the feasibility of the multilayered 3D device. It gives strong potential for high-efficiency and high-throughput biological analysis at low unit cost.

5. Imaging Platforms for Monitoring Cellular Dynamics

As discussed above, the development of label-free biosensors and probe delivery platforms have achieved great success in single-purpose detection both *in vitro* and *in vivo*. Advances in microscopic imaging technologies also boost fundamental research and clinical applications studying complicated dynamics of biological processes [196-198]. Visualization of cellular and subcellular activities, including cell morphology change, cell motility and biomarker expression level variations during biochemical and physiological processes, is critical for disease diagnosis and medicine development.

Conventional microscopies have limited capability to quantify information during live cell imaging. Recent years have seen the rapid development of optical microscopies, e.g. fluorescence microscopy [199], light scattering microscopy [200] and confocal laser scanning microscopy [201, 202], which have become more and more widely used in live cell imaging. In spite of the fact that many optical microscopies serve as valuable tools in cell biology study, concern on cell staining, which is necessary for fluorescence imaging, has raised due to its potential effect on cell viability. Other factors such as photobleaching and phototoxicity have to be considered as well, especially when performing prolonged live cell imaging experiments. In addition to optical imaging techniques, other commonly used cell imaging techniques like scanning electron microscopy (SEM)[203], atomic force microscopy (AFM)[204], etc.,

which do not necessitate molecular labeling (fluorescent or radioactive beacons). To meet the requirement of studying chemical properties of cell membrane, several novel multi-function imaging platforms have been developed, such as scanning electrochemical microscopy (SECM)[205], optogenetic technique[206], photoacoustic tomography (PAT)[207]. Fig. 9 summarizes the road map of imaging platforms for monitoring cellular dynamics. Each of these techniques will be discussed in this section.

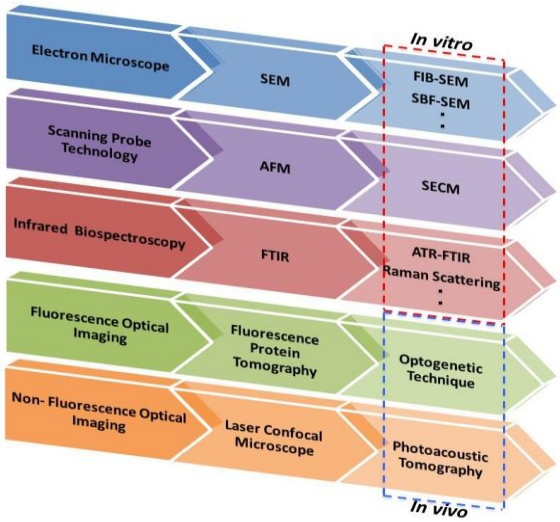

Fig.9 Road map of imaging platforms for monitoring cellular dynamics.

5.1.*In vitro* imaging

Various label-free microscopies have been employed in interrogating cell or bacteria. Electric cell- substrate impedance sensing (ECIS), invented by by Giaever *et al.*[208] was used to measure the temporal change of impedance between gold electrodes that are covered by cell. Since electrical impedance is the function of philological conditions including a tremendous amount of biological and chemical compounds, it is thus employed to quantify cellular activities ranging from cell motility, cell attachment to substrates, to cell morphology changes by interpreting recorded impedance data. But the ECIS cannot give localized information which is related to more detailed subcellular information, as a result of the nature of impedance measurement that averages the results from the area of the size of electrodes containing numerous cells[209].

Wierzbicki *et al.* [210]have presented SEM images with nanoscale resolution successfully showing details of an amount of cell-cell interaction modes on the nanowires fabricated by focused ion beam (FIB) (Fig. 10). Combining FIB-SEM and high resolution (< 5 µm) imaging mass spectrometry (e.g. NanoSIMS), direct imaging also elucidates how the ecological roles of protists and nutrient transfer with symbiotic bacteria [211]. A novel technique, serial block face scanning electron microscopy (SBF-SEM), greatly improved the image resolution and builds three-dimensional (3D) cardiac muscle morphology[212]. Unfortunately, electron microscopies have to use fixed cell samples and thus cannot be used for living cell interrogation, even though it provides desirable spatial resolution.

Scanning probe techniques generally can provide physiological environment for *in vitro* live cell probing localized information without requiring extensive sample preparation, molecular labeling, or high-energy laser beams. Among them, AFM, with up to 1nm lateral resolution, is widely used to investigate membrane-associated structures such as receptors[213], channels[214], exo- and pinocytotic vesicles[215], coated pits[216], microdomains and membrane pores[217]. Malfatti *et al.* have directly measured the cell C in conjunction with AFM-based *Synechococcus* bacteria biovolume[218]. Braet and Wisse have firstly presented a glimpse of fenestrae in living liver sinusoidal endothelial cells (LSECs) [219], as shown in Fig. 10. Recent advances in cantilever fabrication and fast controller development enable high-speed high-resolution live cell imaging using AFM. Notably, AFM imaging usually requires labor-demanding cell sample preparations compared to optical microscopy. In addition, it could induce uncertain force stimulus during cell imaging which may compromise cell viability and cause inaccurate measurement results.

Since it was first invented by Bard *et al.* in 1989, SECM, as a novel scanning probe technique, has provided an attractive tool for studying biological systems, due to its non-contact and label-free working mode. While SECM cannot provide a higher resolution compared to AFM, it possesses an inherent advantage on living cell membrane surface imaging and measurement of electrochemical properties of living cells. Several reviews summarize the theoretical basis, instrumentation and high-resolution SECM (HR-SECM) of living cell specimen[209, 220 221]. For example, Heckman *et al.* have developed a microchip for SECM application in the interrogation of functional myotubes[222]. Shiku and co-workers have quantitatively evaluated the activity of alkaline phosphatase (ALP) using SECM[223], indicating the differentiation progress of embryonic stem cells. In addition, a number of various cell information, including intracellular chemical signals[224], the reactive oxygen species[225], the membrane permeability of nanoparticles[226], and testing controls[227], are successfully interrogated by using SECM. Very recently, Brad *et al.* present a practical application of an analytical strategy that couples micro-3D printing and SECM[228], a versatile platform to manipulate cells and molecules of a population of bacteria with <10 µm precision (Fig. 10). The SECM resolution is mainly limited by probe dimension, tip-to-substrate distance and the property of the substrate. The fabrication of nanometer-scale probes ia pivotal to SECM bio-nanoimaging in the future.

Another technique for examining living cell biochemistry characterization is so-called biospectroscopy. Via infrared (IR) spectroscopy, the resulting spectra, like fingerprints, contain unique information of individual living cells by monitoring the vibrations of chemical bonds within biomolecules [229]. Infrared spectra can be obtained through attenuated total reflection-Fourier transform IR (ATR-FTIR) spectroscopy[230], FTIR microspectroscopy[231] or photo-

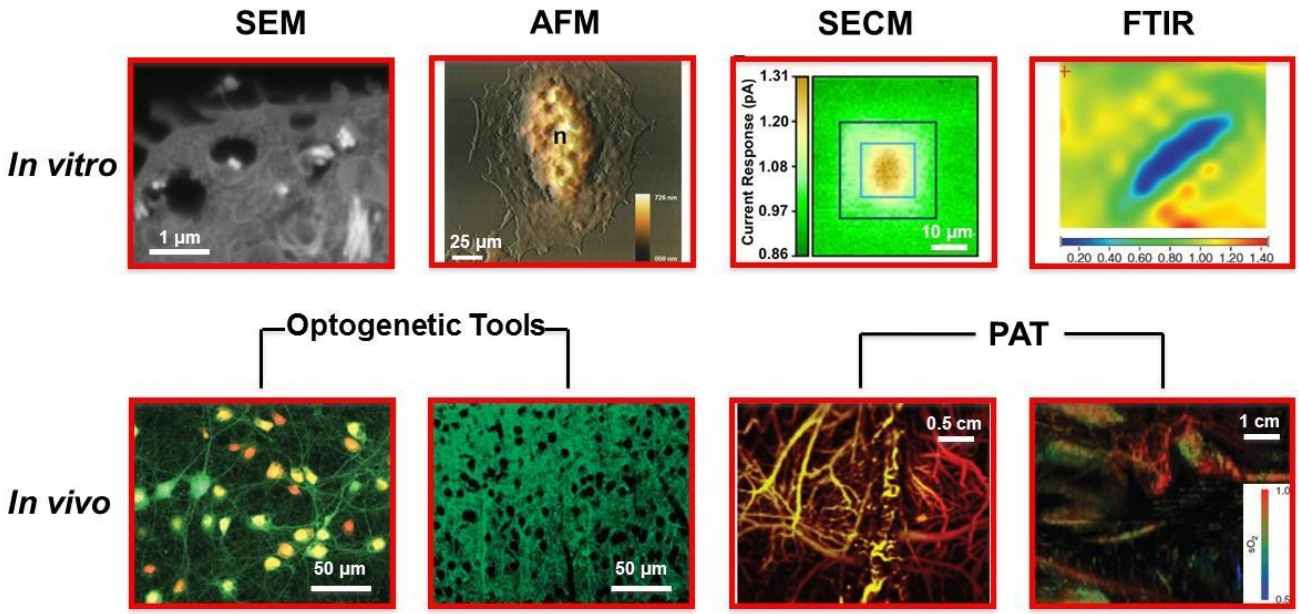

Fig.10 Imaging technologies used in living cell interrogation. Electron microscope (i.e. SEM), scanning probe (i.e. AFM and SECM) and other action spectrum (i.e. FTIR) allow studies of genetic, molecular and cellular events in vitro. Many microscopic imaging technologies have shown significant improvement in the capabilities for anatomical and physiological information acquisition *in vivo*. SEM image reproduced from Ref. 210 with permission from PLOS. AFM image reproduced from Ref. 219 with permission from Elsevier. SECM image reproduced from Ref. 228 with permission from PNAS. FTIR image reproduced from Ref. 235 with permission from Nature Publishing Group. Optogenetic tools images reproduced from Ref. 243 and Ref. 252 with permission from Nature Publishing Group. PAT images reproduced from Ref. 260 and Ref. 264 with permission from Nature Publishing Group.

thermal microspectroscopy[232]. Raman micro-spectroscopy can resolve spectral features by inelastic light scattering sensitive to non-polarized bond, and thus being considered complementary to IR spectroscopy[233]. Martin and co-workers have developed series FTIR biospectroscopy datasets with extracting bio-information[234], distinguishing cell types or populations[235] (Fig. 10), and analyzing biological materials[236]. Comparing to aforementioned methods, FTIR biospectroscopy, especially ATR-FTIR, is a high-throughput imaging technique which can handle a large cell population within e.g., 250 μm by 250 μm area. Today, the challenges of FTIR biospectroscopy are mainly concentrated on sample preparation, instrumentation improvement and efficiency of data processing.

5.2.In vivo imaging

During the last decade, tremendously efforts have been devoted to the use of novel techniques to interrogate the specific cells *in vivo*, and to test whether these cells, regions, and projection pathways are necessary or sufficient for initiating, sustaining, or altering behavior[237-239]. One direct method of recording cellular activity is to analyze the regional electronic signals. Brechet and co-workers designed interpretable behavioral experiments recording electronic signals of single neuron of mouse's brain and simulate the morphology of neurons' grid[240].

In order to obtain direct visualization of spatial cells activities, especially the monitor of neural circuit activity at single cell resolution, optogenetic technology was developed[241]. Amid interpretable optogenetic behavior experiments, optogenetic tools allow for precision control of the electrical activity of genetically targeted neurons by transporting specific ions into/out of cells in response to light. These tools are light-sensitive proteins known as opsins, which are seven-transmembrane proteins that play photo sensory or metabolic roles in species throughout the tree of life[242]. These opsins are responsive to light either by pumping ions into or out of cells, e.g., halorhodopsins pump chloride ions into archaea in response to light[243] bacteriorhodopsins (Fig. 10) and archaerhodopsins pump protons out of archaea in response to light[244], or by opening an ion channel, e.g., channel rhodopsin let cations such as sodium, protons, and calcium into eyespots of algae[245]. By expressing these molecules in specific neurons, regions, or projection pathways, the targeted circuit elements can then be silenced or activated in response to light[246]. There are several reports that have elucidated the principles of designing optogenetic experiments[247, 248], protocol and real-time control of optogenetic interrogation[206], genetically encoded molecular probes for the optogenetic tools[249], *etc*. These opsins have become widespread in neuroscience for the investigation of how specific neural circuit elements contribute to behavior, and are even being contemplated for therapeutic purposes[250].

Recently, LeDoux and co-workers demonstrated that Hebbian and neuro-modulatory mechanisms interact to trigger

associative memory formation in behaving animals using optogenetics[251]. By simultaneously using these complementary techniques, i.e., two-photon excitation of both fluorescence protein and optogenetic tools[252] (Fig. 10), ref-shift optogenetic perturbation tools[253], together with magnetic manipulation[254], researchers are able to interrogate signaling networks and delineate causal relationships between the different molecular players in a more direct way.

The efforts on interdisciplinary collaboration have implemented the emergence of new and innovative imaging platforms. Photoacoustic tomography (PAT), capable of real-time high-resolution molecular imaging deep inside the tissue (~1 mm in human skin), relies on nonionizing radiation without requirement of exogenous imaging agents and have emerged as a useful portable tool in a variety of clinical applications. Images are generated based on the ultrasonic signal from biomolecules. Among different forms of PAT, focused-scanning photoacoustic microscopy (PAM) and photoacoustic endoscopy (PAE) are more often used for deep imaging with micro-scale resolution, and photoacoustic computed tomography (PACT) are good for both microscopic and macroscopic imaging[255]. Wang's group has developed series of optimization algorithm of PAT[256, 257], 2D even 3D imaging of cells[258], organs[259, 260] and vasculatures[261, 262] (Fig. 10). For instance, their PAT is capable of real-time monitoring the anatomical and functional dynamics in blood circulation system [257, 262] by measuring hemoglobin's predominant optical absorption in the visible range. In 2015, quantification of the MRO_2 in a living mouse was reported using this technique [263]. Furthermore, PAT can offer a large field and high spatial resolution when imaging the resting-state nerve system in a living mouse [264] (Fig. 10). Based on endogenous contrast, PAT is also capable of *in vivo* imaging metabolic activities. The scalability of PAT has the potential to link a complex biological system from single cell to the human body [265, 266]. PAT still need to overcome multiple technical challenges to maximize its impact in biomedicine, such as further miniaturization of PAE probe, high-energy lasers for deep-penetration, high-repetition lasers with fast scanning, and novel algorithms for data processing.

6. Conclusion & Prospects

The applications of nanotechnologies in biological and clinical fields have shown great successes in prognosis and long-term monitoring at cellular level. In this paper, we have introduced three representative systems for probing living cells both *in vitro* and *in vivo*. Label-free biosensors have been applied in cell interrogations ranging from extracellular interactions to intracellular functions, receptor signaling, and viral infection. Nano-sensors with the objectives including the function of cellular membrane proteins, cellular action potentials, ion channels, and immunoreceptors are specifically reviewed in the recent 10 years. These novel systems not only claim better spatial resolutions to give an opportunity for single-cell signaling study, but also deeper insight about the functional integration of cell signaling, the receptor signaling pathways, and the action of delivered drug molecules. On the other side, it is worth to note that most of these sensors designed and fabricated are primarily for proof-of-concept, thus being limited to high-throughput with statistical / clinical significance. For better translation from R&D into medical fields, the robust design of the system, mainly depending on the fundamentals and properties of the engineered nanomaterials and nanofeatures, should be improved. Nano-sensors array systems with the capability of massively parallel handling cell-array and multi-parameters detection, present better prototypes than single-sensor platforms with respect to clinical use.

Gene delivery platforms (viral-vectors, chemical and physical) are now widely established in gene therapy, adoptive immunotherapy, wound healing and cancer-treatment, while relatively less trials in probing living cells. A variety of devices with high performance on the high delivery efficiency and precise manipulation of cells are reviewed and highlighted. Chemical nano-carriers are considered as high-throughput non-viral systems and commonly used by medical researchers. Their endocytosis-based delivery results in a few practical issues, including stochastic process, cell-type dependent transfection efficacy, uncertain of cytotoxicity. In comparison, the nanoscale platforms based on physical approaches have demonstrated precise control on genetic manipulation of single-cell, which are still at the stage of proof-of-concept. Filling the gap between the intracellular probe delivery systems and medical requirement require high-throughput designs with promising reliability, portability, repeatability and user-friendliness have to been considered. Integration of continuous cell-flow systems and fabrication of array of functional nanopatterns for massively parallel intracellular delivery, are two commonly recognized designs to achieve high-throughput aims. Moreover, targeted delivery to the intracellular destination (e.g., either cytosol or nucleus), enzymatic resistance and mismatch induced false-positive signals are also promising topics to researchers in non-viral systems.

Microfluidics is one of the mostly recognized approaches for high-throughput cell analysis. Commercial microfluidic-devices have been developed for years. The aforementioned novel microfluidic-platforms, e.g. mass cytometry, 3D droplet microfluidic systems, etc., have shown promising potentials in rapid and accurate detection of biomarkers of cancer cells or stem cells, enzymatic activities, drug screening, etc. They are able to provide high values in implementation of clinical tools for doctors and patients.

Tracking living cell at single-cell level has not been a challenge to modern imaging platforms. Some representative techniques based on AFM, SECM, FTIR, PAT, etc. have demonstrated high resolution for observing the dynamics of ion-channel, nerve-networks or other applications. However, acquisition of molecular information with high resolutions still remains major challenges, especially during the *in vivo* investigation. Novel multi-purpose imaging platforms combining functions of photoacoustic, ultrasound and fluorescence techniques that process comprehensive

diagnostic information need to be development to meet the requirement of the future bioresearch.

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