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Single-molecule and particle detection on true portable microscopy platforms

Lydia Skolrood, Yan Wang, Shengwei Zhang, Qingshan Wei *

Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC 27695

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ABSTRACT

Point-of-care technologies (POCT) that enable early disease detection and therapeutic monitoring are crucial for the next generation of diagnostics and personalized medicine. Meanwhile, there is a global need for low-cost POCT that makes advanced diagnostic tools accessible to resource-limited settings. Recently, several mobile imaging platforms for single-molecule and particle detection have been developed, which greatly improve the detection sensitivity of molecular assays. This review highlights emerging technologies that achieve single-molecule and particle optical detection on true portable platforms. Miniature, high-sensitivity imaging devices based on smartphones, single-board computers (i.e., Raspberry Pi systems), lab-on-a-chip systems, and 3D-printed microscopy platforms are discussed.

1. Introduction

Methods for precision health and medicine have been in the public spotlight for decades, with innovative and advanced diagnostics in laboratory environments complementing strategies for at-home health monitoring. Observation of single molecules represents the highest detectable sensitivity and has been a longstanding goal of analytical methods. With the advancing technologies of modern instruments such as spectroscopy and microscopy with higher sensitivities, optical tools can now detect individual biomolecules such as nucleic acids and proteins at the single-molecule level, powering ultrasensitive diagnostic tests and providing an important understanding of complex biological processes that cannot be elucidated with traditional ensemble techniques [1]. Detection of disease biomarkers from complex clinical samples using single-molecule biosensors can improve patient outcomes through early disease diagnosis and treatment monitoring [1]. Single-molecule detection opens up opportunities to track diseases with ultralow levels of biomarkers [2]. For instance, the selection of molecularly targeted therapies for cancer treatment could be improved by detecting tumor-specific protein biomarkers or circulating nucleic acids at femtomolar concentrations [3,4].

Recent years have seen single-molecule detection playing an increasingly important role in biological science, such as virus particle detection [5], protein dynamics [6], and DNA sequencing [7]. Additionally, single-photon emitters such as single fluorophores are of interest for quantum information systems [8]. However, traditional microscopy and spectroscopy approaches toward single-molecule imaging require bulky and expensive instruments, restricting their use to laboratories and other high-tech facilities. For this reason, researchers have created a variety of innovative point-of-care technologies (POCT) capable of single-molecule and particle imaging (**Fig. 1**). Much effort to develop miniature handheld devices as portable microscopes builds upon a technology that over 48% of the global population [9] already has in their pockets—smartphones [10–12]. Since the launch of the first iPhone in 2007, the popularity of modern smartphones sky-rocketed, and so did innovations in technology for mobile devices. Today's smartphones have high-definition (HD) cameras with megapixel resolutions that can rival high-end photography equipment. Detecting individual nanoparticles and single-molecule events becomes possible by using smartphones and other mobile imaging devices as miniature microscopes [13–16]. Throughout this review, platforms that exist in miniaturized, self-contained, and lightweight form factors will be referred to as "true portable." Many true portable systems for high-sensitivity imaging analysis have been developed in recent years, including devices for imaging individual fluorophores, proteins [17,18], viruses [13], nucleic acids [15], and nanoparticles [13,19] (**Table 1** & **Fig. 2**). Most techniques utilize fluorescence with labels or tags for single-molecule detection. However, it is advantageous for resource-limited settings if the complex biological samples can be analyzed without extensive pre-processing steps. As such, label-free

* Corresponding author.
E-mail address: qwei3@ncsu.edu (Q. Wei).

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methods have also been developed based on holographic or plasmonic techniques, including lens-free holography, surface-enhanced Raman spectroscopy (SERS), and surface plasmon resonance (SPR) methods [20].

The development of portable optical systems with high sensitivity and resolution for single-molecule and particle imaging faces several challenges that are do not present issues in the use of benchtop analysis. Conventional fluorescence microscopes, spectrophotometers, and other non-portable laboratory instruments can rely on building electricity to power scientific-grade lasers, cooled detectors, and other components that enable higher sensitivity than low-powered alternatives used in portable systems (i.e., laser diodes). Although benchtop systems are typically considered too bulky or inconvenient for POCT (**Fig. 1**), the additional space afforded by their larger form factors gives room for more advanced optical and mechanical components (i.e., motorized x,y, z-translation stages, high-magnification objectives, etc.) than can be used in miniature systems. However, the high cost, long result turn-around, and need for operator expertise render the use of conventional benchtop approaches inaccessible to individuals in resource-limited settings that need more affordable and user-friendly options.

The purpose of this review is to highlight the recent development of portable microscopy and spectroscopy systems that achieve single-molecule and particle detection, with a focus on optical sensing devices that are promising as low-cost and ultrasensitive diagnostic tools for use in resource-limited settings. There are several comprehensive reviews that the reader should consider for a broader understanding of the background and recent innovations in developing advanced POCT that use various techniques and detection methods to achieve rapid and accurate results [2,10–12,14,21–26]. Additionally, a recent review by Akkilic et al. gives a clear description of single-molecule biosensors, albeit on non-portable platforms [1]. In addition to optical sensing methods, non-optical methods for single-molecule and particle detection are also in development as POCT, although they are not the focus of this review. For example, solid-state nanopore sensing is seeing rapid growth in recent years for use in DNA sequencing and other single-molecule studies [26–31]. Here, we first highlight various smartphone-based approaches for single-molecule imaging and counting, followed by devices based on 3D-printed lab-on-a-chip technologies that utilize miniature single-board computers (i.e., Raspberry Pi, Arduino) and other off-the-shelf electronics.

2. Smartphone-enabled single-molecule and particle detection

Recent years have witnessed the rise of smartphone-based sensing and imaging systems due to their versatility, accessibility, portability, and cost-effectiveness. Furthermore, smartphones are already equipped with several necessary features for remote data analysis and communications, making smartphone-based devices are especially well-suited as POCT in the era of the Internet of Things (IoT). As the key sensor for smartphone-based optical imaging, sensing, and detection, smartphone cameras have undergone massive technological advances in recent years. Similar to other cameras and imaging systems, smartphone cameras are also comprised of an imaging sensor and optical lenses. Especially, the imaging sensor of smartphone camera plays an important role in determining the imaging performance, such as resolution and sensitivity. Currently, the mainstream smartphone sensor is a complementary metal-oxide-semiconductor (CMOS) sensor. The pixel count is an important parameter of the smartphone camera sensor. In the past decade, the pixel count of image sensors installed on mobile phones doubled almost every two years, following a trend similar to Moore's law [32]. For example, the recent smartphone-based CMOS image sensors (e.g., Sony IMX 586) can provide pixel counts as high as 48 megapixels and pixel size as small as 0.8 μm [33]. As a result, the optical resolution of a smartphone microscope has been significantly improved to the submicron level [34]. Pixel size is another important parameter of an imaging sensor. A larger pixel size usually means more sensitivity as it collects more photons per pixel. Although the pixel sizes of most smartphone CMOS sensor (1–3 μm) are smaller than that of the scientific CMOS camera (>5 μm), the gap is narrowing as the quantum efficiency (70–80%) and read noise level (<2 RMS) of smartphone CMOS sensor is approaching its benchtop counterpart (e.g., EMCCD or sCMOS). These recent advances in imaging hardware have made it possible to take high-quality images with smartphones, which are closely comparable with those obtained by high-end EMCCD/sCMOS cameras. Impressively, smartphone cameras have been transformed into high-resolution microscopes and spectrometers using low-cost device attachments or homebuilt configurations [13,15,16,35,36]. The following section describes the current smartphone-based technologies for optical single-molecule and particle detection.

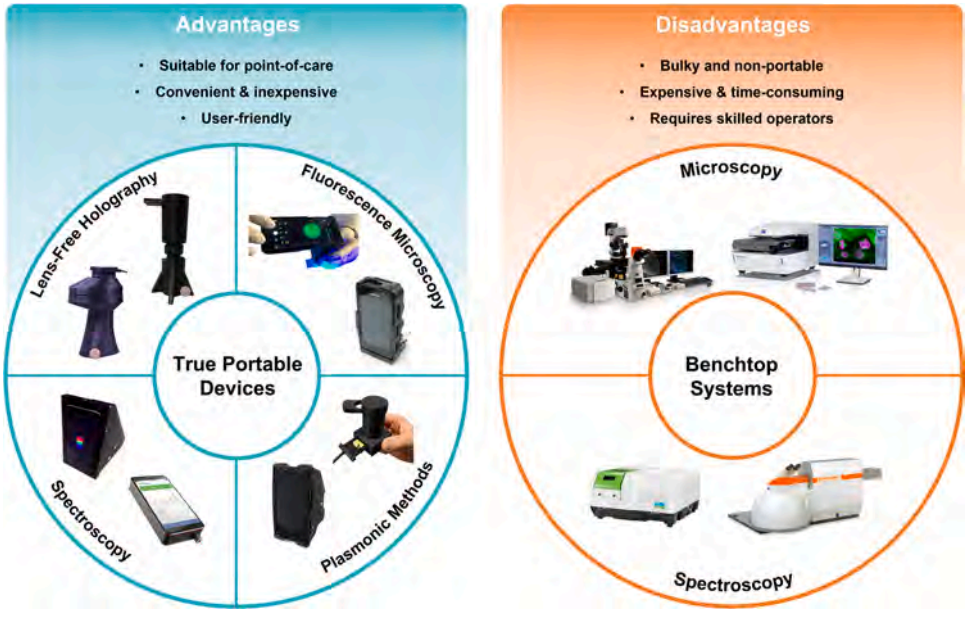

Fig. 1. Comparison of benchtop and portable techniques for single-molecule and particle detection.

2.1. Smartphone-based fluorescence microscopy

Performing single-molecule imaging on smartphones has remained an important objective for researchers. Fluorescence microscopy has been the gold standard for single-molecule detection since it was first put to such use. Naturally, researchers exploring smartphone-based devices for single-molecule and single-particle detection commonly rely on fluorescence-based techniques. For example, research efforts led by Ozcan and coauthors sparked great interest in the development of smartphone-based fluorescence microscopes using various designs. Single-nanoparticle imaging was achieved on a smartphone in 2013 using the built-in complementary metal-oxide-semiconductor (CMOS) imaging sensor and oblique illumination for highly sensitive fluorescence imaging [13]. Wei et al. imaged single nanoparticles and viruses [13] as well as DNA molecules [15] using a smartphone camera as the detector by building a lightweight, 3D-printed optomechanical attachment equipped with a fluorescence excitation source, emission filter, and external lens (**Fig. 3**). Specifically, the device includes a smartphone as the controller and detector, a 405 nm wavelength compact laser diode, a long-pass optical filter to reject the scattered illumination, and a translational stage for focusing. The authors used an oblique angle (75°) of the incident light to minimize background from the excitation source and increase the signal-to-noise ratio (SNR), allowing the handheld microscope to image single cytomegaloviruses (150-to-300 nm) and polystyrene nanoparticles (100 nm) labeled with fluorescent dyes with a spatial resolution of ~1.5 μm [13]. Single-particle imaging was validated with SEM and photon counting (**Fig. 3a**).

This achievement, summarized by Khatua and Orrit in 2013, sparked new interests in the field of low-cost, high-resolution smartphone microscopy [14]. The simple yet effective optical illumination pattern in this work was adopted in subsequent designs for smartphone imaging systems [37–39], including a homemade smartphone-based total internal reflection fluorescence (TIRF) microscope built with plastic bricks, a laser pointer, and other household and hobby-grade items that is capable of accurately measuring single particles of >325 nm in diameter [16]. Fluorescently labeled single DNA molecules were also successfully imaged using a smartphone microscope (**Fig. 3b**) [15]. Different lengths of DNA were stretched into linear segments before being imaged under the smartphone fluorescence microscope. From the smartphone fluorescent image, the length of DNA could be measured, and a linear relationship between lengths measured from smartphone and benchtop microscope has been established. The abovementioned works have successfully demonstrated the applicability of smartphone imaging systems as accessible characterization tools for single-molecule science. Comparable sensitivity with commercial benchtop fluorescence microscope has been tested on smartphone imaging systems. Especially, smartphone imaging of single DNA molecules marks the first case of biomacromolecule imaging using a smartphone [15].

Light-sheet fluorescence microscopy (LSFM), also known as single-plane illumination microscopy (SPIM), was recently demonstrated on a true portable platform for the first time [40]. In SPIM, fluorescence emission is collected perpendicular to a "sheet" of laser light that illuminates only a thin planar volume in the sample located at the focal point of the detector. SPIM methods reduce scattered and out-of-focus light for fast image acquisition with high contrast [41]. The miniSPIM platform is a low-cost, smartphone-based device capable of imaging and

Table 1
Representative examples of single-molecule, -particle, and -virus detection on true portable systems.

Platform	Size	Detection Method	Analyte	Resolution	Field of View	Ref.
Smartphone-based platform	–	Fluorescence microscopy	DNA biomarker for *Klebsiella pneumonia*	–	–	[48]
	17 × 11.3 × 16.8 cm		Single thymine-Hg^{2+}-thymine nucleus acid pairs labelled with fluorescent beads	2.2 μm	1.5 mm^2	[36]
	–		Single intact noroviruses	–	–	[43]
	–		Rolling circle amplification (RCA)-amplified single molecules	0.98 μm	~0.8 mm^2	[44]
	< 190 g		Stretched DNA molecules (~48 kbp)	–	~2 mm^2	[15]
	~186 g		Nanoparticles and viruses	~1.5 μm	~9 mm^2	[13]
Miniature 3D-printed platform	1.9 g		Ca^{2+} spiking and locomotion of Purkinje neurons	~1.5 μm	0.5 mm^2	[65]
	–	Holographic Imaging	Polystyrene nanoparticles	–	~30 mm^1	[19]
	–		Individual protein crystals	–	>10 mm^2	[18]
	< 500 g		Herpes simplex virus (HSV-1) particles	–	~30 mm^2	[70]
	< 500 g, ~25 cm tall		Trypsin nanoparticles	–	–	[68]
	<500 g		Polystyrene particles, Ad5 Adenovirus	–	30 mm^2	[67]
	–		Polystyrene particles, carbon nanotubes	–	>20 mm^2	[66]
	<145 g, 17 × 6 × 5 cm		Pap smear samples	0.87 μm	~21 mm^2	[92]
	~95 g		Patterned microstructures, malaria parasites	< 1 μm	~24 mm^2	[71]
Smartphone-based platform	~12 × 6 cm	Single-plane illumination microscopy (SPIM)	Single fluoresent particles, bacteria, live zebrafish embryo, solvatochromic characterization of solvent polarity	3.1 μm	0.25 mm^2	[40]
	–	Bright-field (BF) transmission, oblique illumination dark-field (OIDF), total internal reflection dark-field (TIRDF) microscopy	Nanoparticles, microbeads, cells	2 μm	~21 mm^2	[42]
	–	Total internal reflection fluorescence (TIRF) microscopy	Polystyrene particles	1 μm	–	[16]
Miniature 3D-printed platform	~ 28 × 15 × 13 cm	Fluorescence, bright-field (BF), cross-polarized microscopy	*P. falciparum* parasites	<775 nm	–	[64]
	~10 × 20 cm	Fluorescence correlation spectroscopy (FCS), Confocal Microscopy	Single proteins, α-synuclein amyloid fibrils using Thioflavin T (ThT), liposomes, and bacteria	–	~1 fL focal volume	[17]
Smartphone-based platform	< 400 g	Dark-field (DF) microscopy	Nanoparticle-based quantification assays	–	–	[35]
	370 g	Surface-enhanced fluorescence (SEF) microscopy	Nanoparticles, quantum dots	–	–	[20]
	~14 × 7.5 × 17.5 cm	Fluorescence spectroscopy, molecular beacon assay	Single base pair mutations in miRNA	–	–	[45]
	–	Transmission spectroscopy with photonic crystal biosensor	Protein monolayer, IgG capture by immobilized Protein A	0.16 nm	750 × 100 pixels	[46]

Fig. 2. Timeline of the development of true portable platforms for single-molecule and -particle analysis. (Reprinted from references [13] [16], [20], [35], ,[40], ,[42], ,[45] ,[48] [59], [66], [72],).

tracking the motion of live cells and single fluorescent particles with image mean square displacement analysis (iMSD). The device uses a battery-powered laser diode, sample cuvette, and optical components (i.e., aperture, cylindrical and aspheric lens, emission filter) mounted on an aluminum plate in front of the smartphone camera that occupies a smaller footprint than the phone itself (**Fig. 3c**). Additionally, implementing image analysis methods to separate the red and green components from the miniSPIM images enabled studies of solvent polarity based on general polarization analysis with a solvatochromic dye. The authors further demonstrated the application of the platform for microbiology and ecology field studies by collecting 3D multichannel and time-lapse SPIM images of live zebrafish embryos [40].

The development of smartphone-based fluorescence microscopes has advanced to multi-modal systems that can incorporate a range of detection methods for adaptive diagnostic strategies based on POC needs. For example, nanoparticles, microbeads, and cells were imaged on a smartphone-based platform for transmission bright-field (BF), oblique illumination dark-field (OIDF), total internal reflection dark-field (TIRDF) microscopy [42]. Additionally, paper microfluidic particulometry was demonstrated on a smartphone-based fluorescence microscope capable of detecting noroviruses at the single-copy level [43]. Point mutation assays, such as the rolling circle amplification (RCA) assay, have also demonstrated their use in achieving sensitive single-base mismatch detection on smartphone-based fluorescence platforms [44].

2.2. *Smartphone-based single-molecule spectroscopy*

In addition to fluorescence microscopy, spectroscopic methods are informative tools that can give chemical or structural information that traditional microscopy cannot. While monochromatic detectors have been shown to increase the sensitivity of fluorescence microscopy, spectral resolution enables single-molecule studies that can identify and differentiate between different chemical species and fluorophores. Yu et al. report the first example of a smartphone-based spectrometer for fluorescence spectroscopy, which is capable of detecting single-base mutations in nucleic acids when paired with a fluorescent molecular beacon assay [45]. In their design, a green laser pointer is focused onto a test sample outside of the enclosed smartphone-based spectrometer, and the resulting fluorescence signal is then guided into an enclosed spectrometer attachment with external optics and an optical fiber cord. After passing through a pinhole, collimator, and cylindrical lens, a transmission diffraction grating (1200 l/mm) placed in front of the smartphone camera resolves the fluorescence signal into its spectral components. With the added benefit of differentiating between fluorophores, smartphone-based spectrometers could be used for multiplexed analysis of fluorophores with different emission wavelengths. Laser diodes with different wavelengths could also be included in such devices to expand the applications of this technology. However, each added component will contribute to the overall weight and bulk of the smartphone-based device, so there is a trade-off between including useful device features and maintaining a small, lightweight form factor.

A non-fluorescence-based approach to smartphone-based spectroscopy has also been developed for detecting protein monolayers and immunoglobulin G antibody capture on a photonic crystal biosensor [46]. The device uses a smartphone camera to image changes in the transmission spectrum of the photonic crystal when biomolecules are adsorbed onto its surface. A broadband light source, pinhole, collimator, polarizer, photonic crystal, cylindrical lens, and grating are enclosed in an aluminum cradle, which holds the smartphone for detection and image display. Label-free approaches such as this allow for simplified sample preparation and rapid analysis compared to methods that require fluorescent tags, which are favorable traits for expanding the use of POCT in resource-limited settings.

2.3. *Plasmonic-enhanced single-molecule detection*

Despite the recent advancements in smartphone-based fluorescence microscopy, imaging single molecules and particles remains a challenge due to the limitation of sensitivity and numerical aperture (NA) of the smartphone camera. Researchers pair plasmonic enhancement methods with fluorescence microscopy to overcome this challenge and achieve single-molecule sensitivity on smartphone-based platforms. In an early example, a thin film of silver was used as the plasmonic substrate to enhance fluorescence signals from DNA-origami nanobeads. The limit of

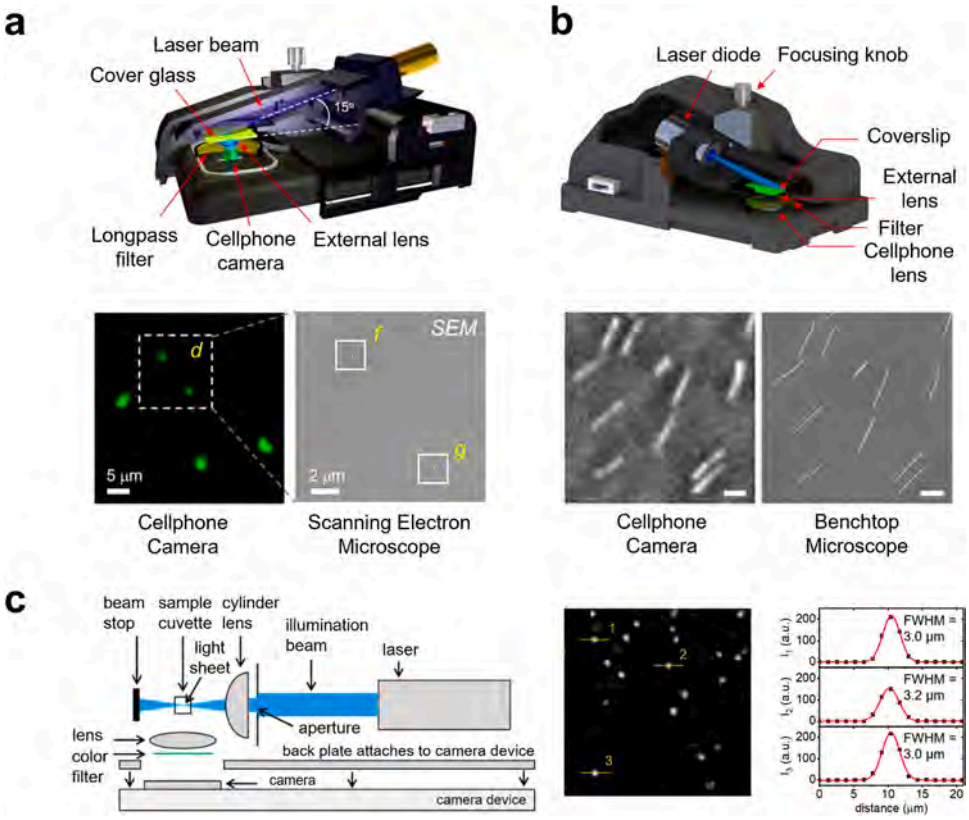

Fig. 3. Representative works on smartphone fluorescence imaging of single particles and biomolecules. (a) Smartphone fluorescent imaging of single 100 nm polystyrene nanoparticles. (b) Smartphone fluorescent imaging and sizing of single DNA molecules (scale bar: 10 μm). (c) Miniaturized light-sheet fluorescence microscope (miniSPIM) imaging on a smartphone and cross-section Gaussian fits of 1 μm polystyrene particles. (Reprinted and modified from ref. [13, 15, 40]).

sensitivity in this work was ~80 fluorophores per diffraction-limited spot [20]. The sensitivity could be further increased to ~10 fluorophores per diffraction-limited spot using an upgraded smartphone model with a monochromic light sensor [47]. More recently, single-molecule blinking and photobleaching events were observed on a standalone, portable smartphone microscope [48]. In this work, strong signal amplification was achieved using silver nanoparticle dimers attached to two DNA origami pillars, also known as NanoAntennas with Cleared HOtSpots (NACHOS) (**Fig. 4a**). Up to 461-fold of fluorescence enhancement was observed. Single-molecule imaging was conducted on a smartphone microscope using a portable laser as a light source and an inexpensive lens module as an objective. Typical single-molecule events like blinking and single-step photobleaching were observed from the transient events extracted from the smartphone video clips. A sandwich DNA detection assay was also built to demonstrate POCT potential. The strongly enhanced fluorescence signal was only observed in the presence of the target DNA sequence, demonstrating the future application of this system on POC diagnostics.

In addition to plasmonic methods for enhanced fluorescence detection, surface-enhanced Raman spectroscopy (SERS) utilizes plasmonic materials to enhance Raman signals and enable detection down to the single-molecule level [49–52]. SERS substrates are comprised of plasmonic nanostructures, usually silver or gold nanoparticles. Previously, single-molecule SERS analysis was thought to require enhancement of the Raman signal by a factor of 10^{14}, but it has since been determined that an enhancement factor (EF) as low as 10^{7} is sufficient for single-molecule sensitivity [53–55]. Single-molecule blink events can be imaged with SERS using super-resolution label-free methods [56,57]. A smartphone-based Raman spectrometer that demonstrates single-molecule counting has been achieved in non-portable systems confined to laboratory settings [57]. In this work, single-molecule blinking events were observed on the smartphone by using Ag nanoisland on Ag overlayer and HfO_2 dielectric layer as the enhancement substrate (**Fig. 4b**). The authors used a modified configuration of a commercial confocal Raman microscope that allows the smartphone CMOS camera to capture the SERS signal. The modified instrument was equipped with fiber optic SERS collection, a source filter, another fiber optic cord that acts as a slit, and a collimation lens, before passing through a transmission diffraction grating for spectrally resolved detection with a smartphone CMOS camera. Single-molecule blinking events were observed from smartphone videos recorded at 30 fps with the optimized plasmonic substrate. By comparing the time series of Raman spectrum between the smartphone detector and a cooled CCD detector, similar single-molecule blinking events were observed, indicating that single-molecule events were observed on a smartphone camera coupled with laboratory equipment [57]. Later, a true portable smartphone-based SERS device [58] equipped with cloud network architecture [59] was developed for use in pesticide and pollutant sensing [58,60–62] and disease diagnosis [63]. Instead of utilizing the smartphone CMOS camera, the reported device uses a CCD detector in the compact SERS attachment that is coupled to the phone via a data port (**Fig. 4c**). This device has been paired with SERS-enabled LFA techniques for sensing inflammation biomarkers [63] and low-cost paper--based plasmonic chips for portable detection of pesticide residues as low as 10 ppm [62]. However, to the authors' knowledge, this true portable smartphone-based SERS device has not been used to observe time-dependent Raman blinking events or other single-molecule studies.

3. Miniature microscopy devices for single-molecule and particle imaging

Recent years have witnessed a community-driven effort toward creating highly integrated, portable, and low-cost biosensing instruments. Other than smartphone-based platforms, miniaturized setups

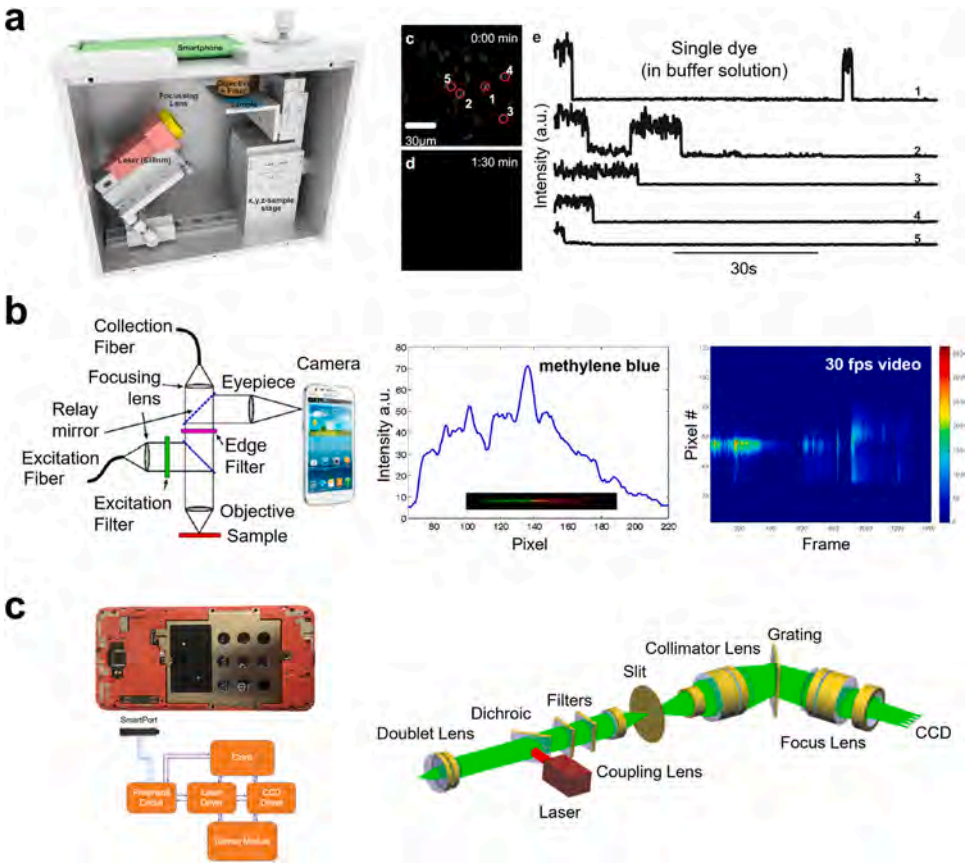

Fig. 4. Devices for smartphone-based single-molecule detection using plasmonic enhancement, including (a) a smartphone-based fluorescence microscope utilizing addressable NACHOS and DNA origami for imaging single fluorophores and (b) a non-portable SERS detection system using a smartphone camera to detect single-molecule spectral blinking events of 10 nM methylene blue dye solution on plasmonic substrates at 30fps. (c) A true portable smartphone SERS platform using a compact spectrometer and CCD sensor for Raman detection. (Reprinted and modified from ref. [48, 57, 59]).

built with off-the-shelf consumer electronics, optical components (i.e., lenses, filters, collimators, etc.), and 3D-printed supports have been developed, including both lens-based [17,64,65] and lens-free [18,19, 66–72] systems. Various types of analytes (i.e., cell-based assays [22,64, 73–77], bacterial or viral pathogens [69,70,78–80], proteins [72,81–83] and nanoparticles [67,68,84–86]) have been imaged and detected on these miniaturized devices.

3.1. *Fluorescence microscopy*

Many of the early fluorescence-based miniaturized systems were designed for single-cell imaging applications. For example, Ghosh et al. fabricated an integrated fluorescence microscope for high-speed cellular imaging in live mice brain models [65]. Gordon et al. designed a portable multi-modal microscope that can take bright-field, fluorescent, and cross-polarized images by manually changing the filters [64]. More recently, a miniaturized modular-array fluorescence microscope was reported for possible parallelization of multi-site, live-cell imaging in 8-well plates [87]. The portable up-right modular architecture allows time-lapse in situ live-cell imaging and analysis inside a conventional incubator.

Compared to single-cell imaging, small molecules like proteins or nanoparticles are challenging to observe under miniature fluorescence microscopy due to their low emission intensity, which requires super-resolution or signal enhancement for sensitive and accurate quantifications. Singh et al. developed an instrument for fluorescence correlation spectroscopy (FCS) with a resolution of 215 nm using an avalanche photodetector (APD) in a compact form factor [88]. The authors demonstrated single-molecule studies, using the instrument to measure the diffusion time of rhodamine B dye in an aqueous solution. However, since the design uses an external source, detector, and data display with a desktop computer confining it to benchtop settings, it is not considered true portable for this review. More recently, Brown et al. demonstrated a compact, low-cost single-molecule confocal system, the AttoBright, for POC detection of single proteins, protein aggregates, liposomes, and bacteria [17]. On this setup, the use of a high-end objective, a simplified optical path, and optimized optical components enables the high performance required for single-molecule detection (**Fig. 5**). Unlike most portable platforms using CMOS imagers, this miniature confocal microscope utilized a single-photon counting APD module, providing higher sensitivity for single-molecule detection. The single-photon APD has a 50 μm diameter active area, which is used as a pinhole, further reducing the number of optical elements required on a general confocal microscope. In order to direct emitted light onto the active area of the detector, optical alignment is performed by adjusting the scanning mirror mounted on piezoelectric motors (**Fig. 5a**), which is driven to move stepwise on x-y dimensions by a Labview program in a feedback manner. Within a fixed experimental volume, individual molecules are detected as they diffuse in and out of the focal volume such that the photon counts per molecule and the average number of molecules within the observation volume can be extracted by the photon counter (**Fig. 5c**). The authors demonstrated various experimental applications on this platform, including protein-micelle interactions, liposome disruption, bacterial detection, and pathological protein aggregate detection. The latter example showed sensitivity to 15 pg/ml, 10^6-fold more sensitive than bulk detection and comparable to more complex single-molecule systems.

3.2. *Lens-free holographic methods*

Lens-free on-chip microscopy is another portable platform that has been explored for single-molecule detection. In 1994, Lamture et al. reported a lens-less approach with a very high detection sensitivity toward nucleic acid hybrids [89]. The authors placed a hybridization

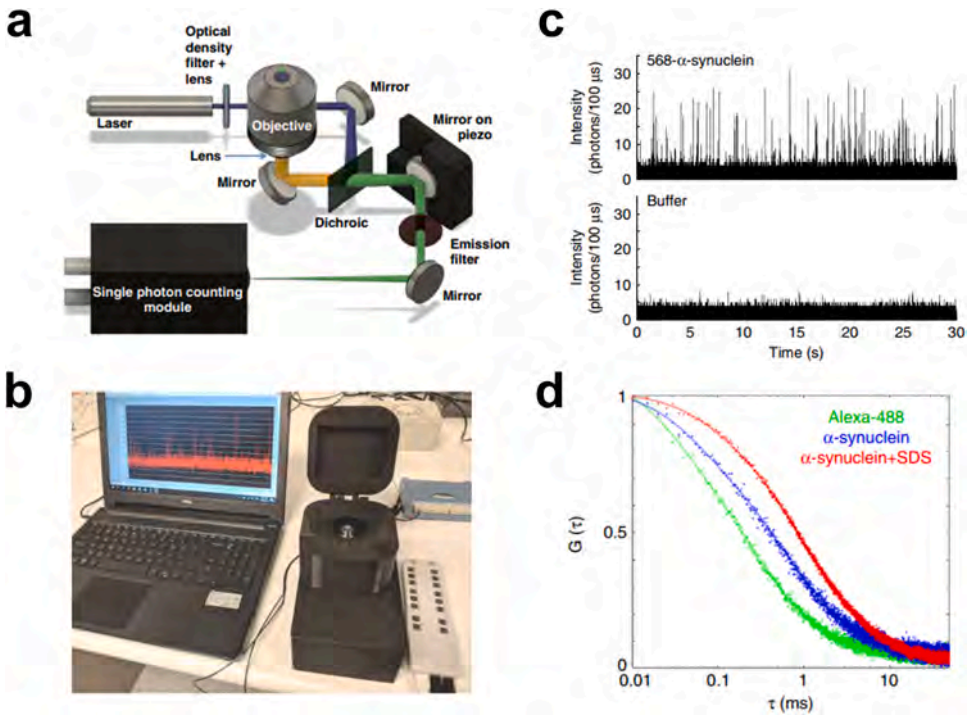

Fig. 5. Design and characterization of a 3D-printed fluorescence correlation spectroscopy platform. (a) Overview of the optical path. (b) Photograph of the Atto-Bright platform connected to a laptop for data acquisition. (c) Detection of single α-synuclein protein molecules fluorescently labeled with Alexa-488, and (d) FCS spectra of 10 nM Alexa-488 fluorophore, labeled α-syn protein, and labeled α-syn protein in the presence of a surfactant SDS. (Reprinted and modified from ref. [17]).

matrix directly upon the surface of a CCD microdevice for detecting beta emission from ^{32}P-labelled DNA. With recent advances in optoelectronic technologies, miniaturized lens-less holographic imaging devices for POC diagnostics have been developed [90]. In a lens-free holographic microscope, the sample is placed above an image sensor chip with a spacing of <1 mm, a coherent or partially coherent light source illuminates the sample from the top. The light source in a portable device is usually a light-emitting diode (LED) with an optional spectral filter to fine-tune the temporal coherence at the sensor plane [91]. As a result, the sample casts an in-line hologram, which is directly recorded by a CMOS or CCD image sensor. From this recorded hologram, the original object, both its amplitude and phase images, can be

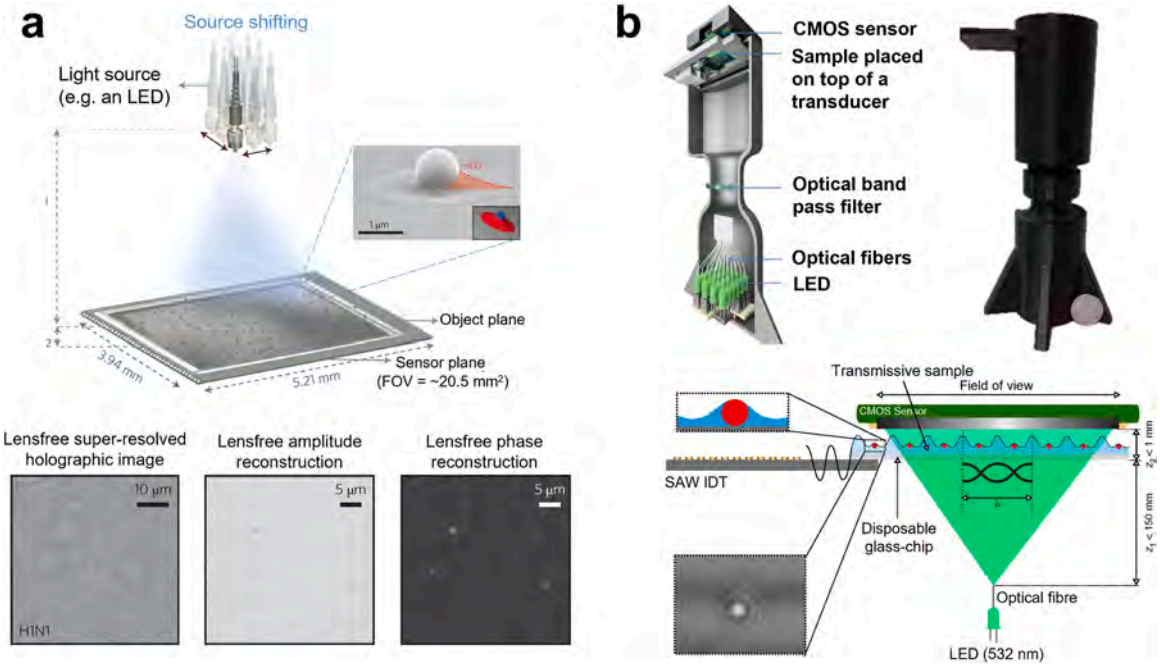

Fig. 6. Representative works on lens-free holographic imaging systems. (a) Wide-field on-chip microscopy with self-assembled nanolenses. Upper panel: Lens-free pixel super-resolution holography schematic. Lower panel: Detection of individual H1N1 viruses. (b) Holographic detection of nanoparticles using acoustically actuated nanolenses. Upper panel: Schematic of the lens-free sensor system and the photography of the 3D-printed physical hardware. Lower panel: Optical system design and schematic of generation of acoustically actuated nanolenses. (Reprinted and modified from ref. [86] and [85]).

reconstructed digitally, where the latter can especially be of use for better visualization of weakly scattering objects such as parasites or pathogens. Image processing techniques can be used to remove image artifacts and further improve the resolution to the sub-micron level in partially-coherent digital in-line holography [92].

Ozcan's group developed several miniaturized holographic microscopes for imaging nanoparticles [66–68,85], proteins [18], bacteria and virus [70,79] and parasites [71] in the last decade. They designed a holographic lensless microscope integrated with an array of 23 multi-mode fibers, which are butt-coupled to 23 LEDs, respectively. Each LED is sequentially turned on using an inexpensive micro-controller such that the sample is illuminated by a single fiber at a given moment, creating lens-free holograms of the objects on a CMOS sensor array. These recorded lens-free holograms are shifted with respect to each other and can be rapidly processed using a pixel super-resolution algorithm to create transmission images of the objects achieving a wide FOV of ~24 mm^2 with a resolution less than 1 μm [71].

By using a biocompatible wetting film to self-assemble aspheric liquid nanolenses around individual nanoparticles, the contrast between the scattered and background light can be enhanced, allowing sub-100 nm particles across a large field-of-view (FOV) of >20 mm^2 to be detected based on the holographic diffraction patterns (**Fig. 6a**) [86]. On this compact on-chip microscopy, the authors have demonstrated the detection of individual polystyrene nanoparticles, adenoviruses, and influenza A (H1N1) viral particles (**Fig. 6a**). More recently, instead of generating the self-assembled nanolenses by tilting the plasma-treated glass coverslip to disperse the nanoparticles, an ultrasonic standing wave is used to create the lens-like liquid menisci around the individual particles (**Fig. 6b**) [85]. By creating an ultrasonic standing wave in the liquid sample placed on a low-cost glass chip, deformations were generated in a thin liquid layer (850 nm) containing the target nanoparticles (≥140 nm). This effect results in the creation of localized nanolens around the nanoparticles and enhances their optical signal response. This acoustically actuated lens-free holographic microscopy demonstrates a larger FOV of 30 mm^2 but the same sub-100 nm detection capabilities.

3.3. Plasmonic methods

Surface plasmon resonance (SPR) is another diagnostic scheme that has been extensively used for the detection of single molecules or nanomaterials [93]. Plasmonic resonance is a unique optical property of metallic nanostructures generated when their dimensions are smaller or comparable to the wavelength of incident light. Because of the tunability and large electromagnetic (EM) enhancement effect, SPR-based techniques can be used for sensitive detection of analytes such as protein immunoassays [94] and nucleic acid biomarker [95], and thus hold revolutionary potential in POC biosensing.

Altug's group reported an optofluidic-nanoplasmonic sensor for fast, compact, quantitative, and label-free sensing of viral particles. The nanohole array-based sensing platform used antiviral immunoglobulins immobilized at the sensor surface for specific capturing of different types of viruses (VSV, PT-Ebola, and Vaccinia) in the biological media, and the concentrations were quantified on this platform [96]. Later, Ozcan and Altug together developed a handheld plasmonic biosensor by coupling plasmonic Au nanohole arrays with a lens-free on-chip imaging system for high-throughput screening of biomolecular binding events. This portable biosensor is able to detect protein monolayers down to 3-nm thickness without any labels and enables quantitative analysis of protein binding events over a wide range of biomolecule concentrations (**Fig. 7c**) [69,72]. More recently, Altug et al. also used Au nanohole arrays as sensing substrate, but they employed the phase response of the plasmonic resonances for protein detections (**Fig. 7a**) [97]. SPR platforms integrated with microfluidic chips have been developed with the advantage of inexpensive fabrication, adaptability, and rapid results. Microfluidics also helps to handle small sample volumes, allowing small drug quantification in patient samples at the POC. For example, a small molecule, tobramycin, can be directly detected in undiluted blood serum via a simple handhold LSPR system integrated with a microfluidic chip, which is composed of a glass slide coated with gold nanoislands (NIs) and functionalized with DNA aptamers (**Fig. 7b**) [98]. Other plasmonic techniques on miniaturized devices are promising for future achievements in optical single-molecule and particle detection. Although several commercial devices for miniature or handheld SERS analysis are on the market, they are expensive (>10k USD) and have yet to demonstrate single-molecule resolution.

4. Conclusion and future perspectives

The recent advancements in POCT are promising for bringing

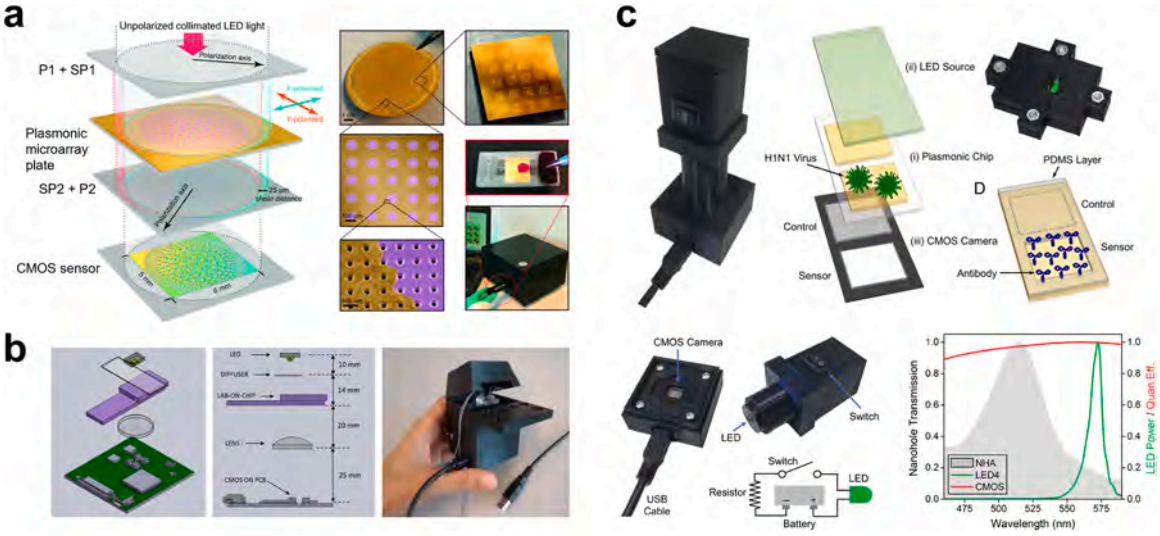

Fig. 7. Representative works on portable plasmonic biosensing devices. (a) Large FOV interferometric microarray imager (LIM) and experimental. Left panel: Collinear optical light-path configuration of the LIM setup. Right panel: Silica microarrays on uniformly patterned plasmonic Au-NHAs and the portable interferometric microarray imager integrated with a disposable capillarity-based microfluidic platform assembled on the plasmonic microarray plate. (b) A portable, palm-sized transmission-localized surface plasmon resonance (T-LSPR) setup with the schematic and photographic of the complete setup. (c) Lens-free plasmonic nanohole array device on a porTable 3D-printed platform for detecting H1N1 virus particles. (Reprinted and modified from ref. [97, 98], and [69]).

precision healthcare tools to resource-limited settings. While techniques for single-molecule imaging on true portable platforms are still in their infancy, several devices have been reported for single-molecule and particle fluorescence microscopy and spectroscopy on smartphones and other mobile imaging devices. Portable technologies based on miniature single-board computers (i.e., Raspberry Pi, Arduino) and lab-on-a-chip technologies that use fluorescence microscopy as well as plasmonic-enhancement methods, lens-free holographic imaging, and confocal microscopy have also been reported to detect single molecules and particles on true portable platforms. Still, there is ample opportunity to further improve the sensitivity and resolution of these POCT and expand across a wider variety of sensing targets by implementing new signal enhancement and detection strategies. For example, future research may develop miniature or portable platforms for super-resolution fluorescence microscopy, which exceeds the theoretical diffraction limit for optical imaging. Additionally, new sensing platforms that pair fluorescence microscopy with spectroscopy and plasmonic enhancement methods have been of interest recently because they can increase sensitivity and provide chemical identification and multiplex sensing capabilities.

POCT for disease diagnostics that have high sensitivity and portable configuration are in extremely high demand, especially since the beginning of the COVID-19 pandemic. Hundreds of publications have been reported on bio-sensing methods developed for SARS-CoV-2 since April 2020, some of which are using direct and rapid paper-based immunoassays for amplification-free DNA or RNA biosensing [99]. By using a smartphone [100,101] or a miniaturized device as the optical detector, COVID-19 diagnostics can achieve detection of single nucleic acids at the POC. Furthermore, connection through the 5 G network enables POCT for COVID-19 to become part of the IoMT, or Internet of Medical Things [102]. It is worth noting that nucleic acid detection with fluorescence microscopy on smartphones and other mobile platforms achieve sensitivity down to attomolar concentrations by means of molecular amplification assays and other methods [39,103–105]. Meanwhile, the focus of this review is to highlight direct optical detection of single molecules and particles, which we distinguish from POCT based on molecular amplification and single-molecule digital assays. Lab-on-a-chip approaches that show promise for use in true portable POCT also include optofluidic techniques such as antiresonant reflecting optical waveguide (ARROW) paired with microfluidic devices [106]. Microfluidics is a great benefit to portable optical detection using fluorescent tags because it enables rapid mixing, purification, and other liquid sample processing to be performed conveniently on small volumes in field settings. Portable microscopy and spectroscopy platforms could soon be paired with optofluidic chips for immunoassays [107], optical waveguide-based spectroscopy [108], or other methods for rapid and early disease diagnosis at the POC.

As the gold-standard technique, fluorescence-based detection methods will likely continue to dominate the field of single-molecule and particle sensing. However, label-free and non-fluorescent methods are preferable for single-molecule studies because of the influence of label molecules on the behavior of the molecule of interest [1,109]. Another benefit to label-free analysis is the reduced requirement for sample processing steps which could be inconvenient or unavailable in resource-limited settings. Plasmonic sensing methods have been used to complement or even replace traditional unenhanced fluorescence microscopy to achieve label-free optical single-molecule and single-particle detection on true portable platforms. As such, true portable devices based on label-free methods, including SPIM, lens-free holography, and plasmonic nanohole arrays, were discussed in this review. However, further research is needed in areas such as smartphone-based SERS to demonstrate single-molecule detection on a true portable platform. The past decade has seen several advancements in plasmonic materials for label-free SERS analysis that are well-suited for POC applications and could potentially bring single-molecule sensitivity to portable SERS platforms [110]. Other methods, such as smartphone-based miniSPIM, have achieved this level of sensitivity but could benefit from employing complementary techniques to increase spatial resolution. For example, using digital scanning light-sheet microscopy (DSLM) with a focused laser beam in a thin planar path instead of a continuous planar beam while collecting the fluorescence image reduces the time that a particular spot on the sample is exposed to laser light, allowing for faster image acquisition which reduces photobleaching effects and improves the signal-to-noise ratios [111]. Furthermore, structured-illumination (SI), confocal, multi-view, and hyperspectral techniques applied to SPIM further reduce the effects of light scattering background in the fluorescence images of complex specimens [41].

Conventional benchtop techniques for high-resolution label-free imaging continue to be translated to novel platforms with innovative miniaturized designs for POC sensing applications. For example, a promising new smartphone-based platform for miniaturized Fourier ptychographic microscopy (FPM) uses the phone's screen for programmable illumination and the front camera for imaging [112]. This low-cost platform, which demonstrates sub-micron resolution, could easily be extended to label-free single-particle imaging studies in the future. Other label-free methods that show great promise as POCT for single-molecule or particle detection but have not yet been explored as such include interferometric scattering (iSCAT) or interferometric scattering mass spectrometry (iSCAMS) [113].

It is important to note that while the many platforms discussed in this review are portable and physically suitable as POCT, affordability is also an important factor when considering technologies for resource-limited applications. Currently, SERS and other plasmonic-based methods for single-molecule detection require nanostructured substrates with large enhancement factors that are made of costly materials and rely on cleanroom fabrication [57]. The need for more cost-effective plasmonic materials has driven research in graphene oxide-based plasmonics for SERS and SPCE methods paired with portable detectors [114,115]. The most affordable solutions may be to upcycle smartphones into fluorescence microscopes using the built-in phone camera, low-cost optical components, and a 3D-printed [17] or homemade frame [16].

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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