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Expansion mini-microscopy: An enabling alternative in point-of-care diagnostics

Yu Shrike Zhang[a,b,c], Grissel Trujillo-de Santiago[a,b,d,e], Mario Moisés Alvarez[a,b,d,e], Steven J. Schiff[f,g,h,i], Edward S. Boyden[j,k,l,m,n] and Ali Khademhosseini[a,b,o,p]

Diagnostics play a significant role in health care. In the developing world and low-resource regions the utility for point-of-care (POC) diagnostics becomes even greater. This need has long been recognized, and diagnostic technology has seen tremendous progress with the development of portable instrumentation such as miniature imagers featuring low complexity and cost. However, such inexpensive devices have not been able to achieve a resolution sufficient for POC detection of pathogens at very small scales, such as single-cell parasites, bacteria, fungi, and viruses. To this end, expansion microscopy (ExM) is a recently developed technique that, by physically expanding preserved biological specimens through a chemical process, enables super-resolution imaging on conventional microscopes and improves imaging resolution of a given microscope without the need to modify the existing microscope hardware. Here we review recent advances in ExM and portable imagers, respectively, and discuss the rational combination of the two technologies, that we term expansion mini-microscopy (ExMM). In ExMM, the physical expansion of a biological sample followed by imaging on a mini-microscope achieves a resolution as high as that attainable by conventional high-end microscopes imaging non-expanded samples, at significant reduction in cost. We believe that this newly developed ExMM technique is likely to find widespread applications in POC diagnostics in resource-limited and remote regions by expanded-scale imaging of biological specimens that are otherwise not resolvable using low-cost imagers.

Addresses
[a] Biomaterials Innovation Research Center, Division of Engineering in Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge 02139, MA, USA
[b] Harvard-MIT Division of Health Sciences and Technology, Cambridge 02139, MA, USA
[c] Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston 02115, MA, USA
[d] Centro de Biotecnología-FEMSA, Tecnológico de Monterrey at Monterrey, CP 64849, Monterrey, Nuevo León, Mexico
[e] Microsystems Technologies Laboratories, Massachusetts Institute of Technology, Cambridge 02139, MA, USA
[f] Center for Neural Engineering, The Pennsylvania State University, University Park 16802, PA, USA
[g] Department of Neurosurgery, Penn State College of Medicine, Hershey 17033, PA, USA
[h] Department of Engineering Science and Mechanics, The Pennsylvania State University, University Park 16802, PA, USA
[i] Department of Physics, The Pennsylvania State University, University Park 16802, PA, USA
[j] Media Lab, MIT, Cambridge 02139, MA, USA
[k] Department of Biological Engineering, MIT, Cambridge 02139, MA, USA
[l] McGovern Institute, MIT, Cambridge 02139, MA, USA
[m] Department of Brain and Cognitive Sciences, MIT, Cambridge 02139, MA, USA
[n] Center for Neurobiological Engineering, MIT, Cambridge 02139, MA, USA
[o] Department of Bioindustrial Technologies, College of Animal Bioscience and Technology, Konkuk University, Hwayang-dong, Gwangjin-gu, Seoul 143-701, Republic of Korea
[p] Department of Physics, King Abdulaziz University, Jeddah 21569, Saudi Arabia

Corresponding authors: Boyden, Edward S (esb@media.mit.edu); Khademhosseini, Ali (alik@bwh.harvard.edu)

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Introduction

Diagnostics play a significant role worldwide by providing proper and timely healthcare to patients [1]. The role of diagnostics and in particular, the need for point-of-care (POC) diagnostics, is even more critical in the developing world and low-resource regions [1–3]. This has been recognized for long, and diagnostic technology has seen tremendous progress with the development of, in particular, imaging-based strategies.

Although conventional optical microscopy provides high-end capacities in terms of resolution, often times their bulky sizes, expense, and need for alignment and adjustment cannot satisfy the need of portable observations. Recent advances in POC diagnostics have further challenged existing microscopy techniques where *in situ* and cost-effective detection is required in addition to sufficient sensitivity and resolution. For example, compact image sensors are widely applicable to POC analysis at mammalian cell level [4–9]. The mini-microscopy has several key advantages over conventional

microscopy in undeveloped regions [10], including: *i*) simplicity, light weight, and compactness: a mini-microscope can be easily constructed with simple design, making the system compact and portable, well suited for POC diagnostics; *ii*) cost-effectiveness: the main components of a mini-microscope include off-the-shelf items such as a light-emitting diode (LED) and an image sensor, making it affordable in POC diagnostics. Nevertheless, while the mini-microscopes are widely applicable in portable POC diagnosis at the scale of mammalian cells, it remains challenging for their utility in detecting disease-causing pathogens (*e.g.* parasites, bacteria, viruses, and fungi) due to the limited resolution of their equipped low-end optics and/or sensors.

Expansion microscopy (ExM) is a recently developed technique that enables super-resolution imaging (*e.g.* below the diffraction limit) on conventional microscopes and improves imaging resolution of a given microscope without the need to modify the existing microscope hardware. ExM relies on a very simple principle, *i.e.* embedding a biological sample in a matrix of swellable polymer, which chemically anchors fluorescent labels already pre-applied to the specimen, and then physically expands the specimen by swelling the specimen-matrix composite to ~4–5 times in linear dimension [11]. Variations of ExM have been further developed recently to permit the simple, powerful anchoring and separation of proteins and nucleic acids of biological samples [12,13].

As such, the rational combination of ExM and mini-microscopy may potentially push forward a new initiative in POC diagnostics, which we term expansion mini-microscopy (ExMM) [14]. We have thus demonstrated that even a low-cost mini-microscope could image biological samples (both mammalian and bacterial cells) at unprecedentedly high resolution comparable to conventional microscopy without expansion, indicating that the integration of the original version of ExM with a mini-microscope through our ExMM technology can result in resolution that rivals that of a conventional microscope with a hundred-to-thousand-fold reduction in the cost. Here in this Opinion Article, we review recent advances in ExM and portable imagers, respectively, and discuss the preliminary findings of their combinatory form, the ExMM. We finally conclude with future perspectives on the potential application of ExMM in POC diagnostics.

Expansion microscopy (ExM)

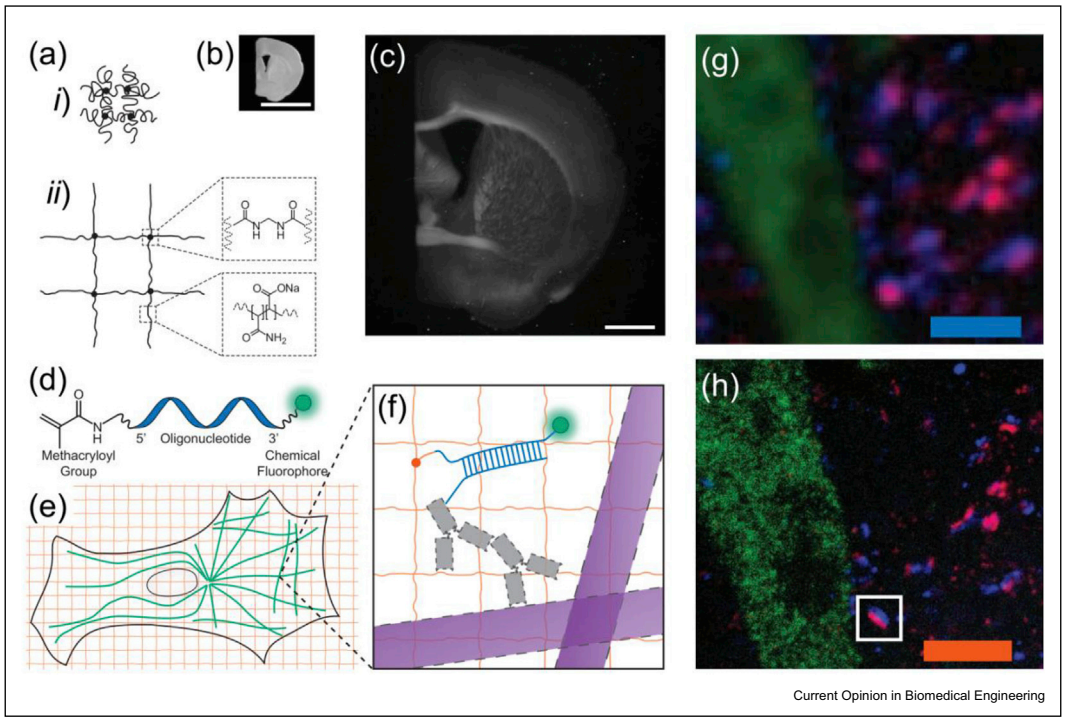
Conventional imaging of specimens with high spatial precision requires expensive equipment, because precise magnification with a minimum of aberration and a maximum of sensitivity requires high-end lenses and cameras, as well as precision alignment. We recently developed a new strategy depending primarily on chemistry to do the magnification – physically instead of optically [11]. We discovered that, by synthesizing a swellable polyelectrolyte hydrogel network directly within a specimen of interest, and subsequently dialyzing the sample in a medium of lower osmolarity (*i.e.* pure water), it could be physically expanded (Figure 1a–c). Specifically, we used sodium polyacrylate as the hydrogel material, which when synthesized in high salt is compact, but when the salt is diluted undergoes swelling due to electrostatic repulsion (Figure 1ai–ii). By staining a sample with a trifunctional label comprised of an antibody, a polymer-linking group, and a fluorophore, we were able to anchor the fluorophore to the hydrogel network (Figure 1d–f); by enzymatically digesting the endogenous structure, we were able to render the sample mechanically homogeneous. Water, then, swells the sample (Figure 1b *versus* 1c). Embedding preserved biological specimens in hydrogels for microscopy imaging purposes has a long history, dating back to 1995 [15], but expansion microscopy uses a specific property of hydrogels – in particular polyelectrolyte gels – namely the massive swelling of such hydrogel upon exposure to pure water, to physically move apart components of a biological specimen, so that nanoscale structures can be resolved.

Since the sample preparation procedures involve enzymatic homogenization of the mechanical characteristics of the tissue-polymer composite, this technology design enables isotropic and uniform expansion to occur [11]. Comparing images pre-*versus* post-expansion, using conventional (structured illumination-based) super-resolution microscopes, in fixed cultured HEK cells as well as in mouse brain slices, we confirmed that this expansion process was isotropic [11], and thus a ~4.5-fold expansion enabled an effective resolution of 300 nm (the diffraction limit of the lens used) divided by 4.5, or approximately 60 nm, approaching that attainable with classical super-resolution microcopy methods – but without requiring the hardware [16,17]. ExM has been utilized in super-resolution imaging of a variety of biological specimens using conventional optical microscopy, including for example, cultured mammalian cells [11], brain tissues [11], and cancerous tissues [18]. As shown in Figure 1, g and h, a Thy1-YFP-H mouse cortex slice was stained with antibodies against yellow fluorescent protein (YFP, green), as well as the pre- and post-synaptic scaffolding proteins Bassoon (blue) and Homer1 (red). The post-ExM image (Figure 1h) showed clear demarcations between the Bassoon/Homer1 signals while in the pre-ExM image (Figure 1g) the staining formed overlapping spots at each synapse due to blur by diffraction.

Further development of ExM

ExM is a chemistry that is easily customized for new applications. The original ExM method was unable to retain native proteins in the hydrogel and used specially designed reagents not widely available [11]. We subsequently developed a variant of ExM, named protein-

Figure 1

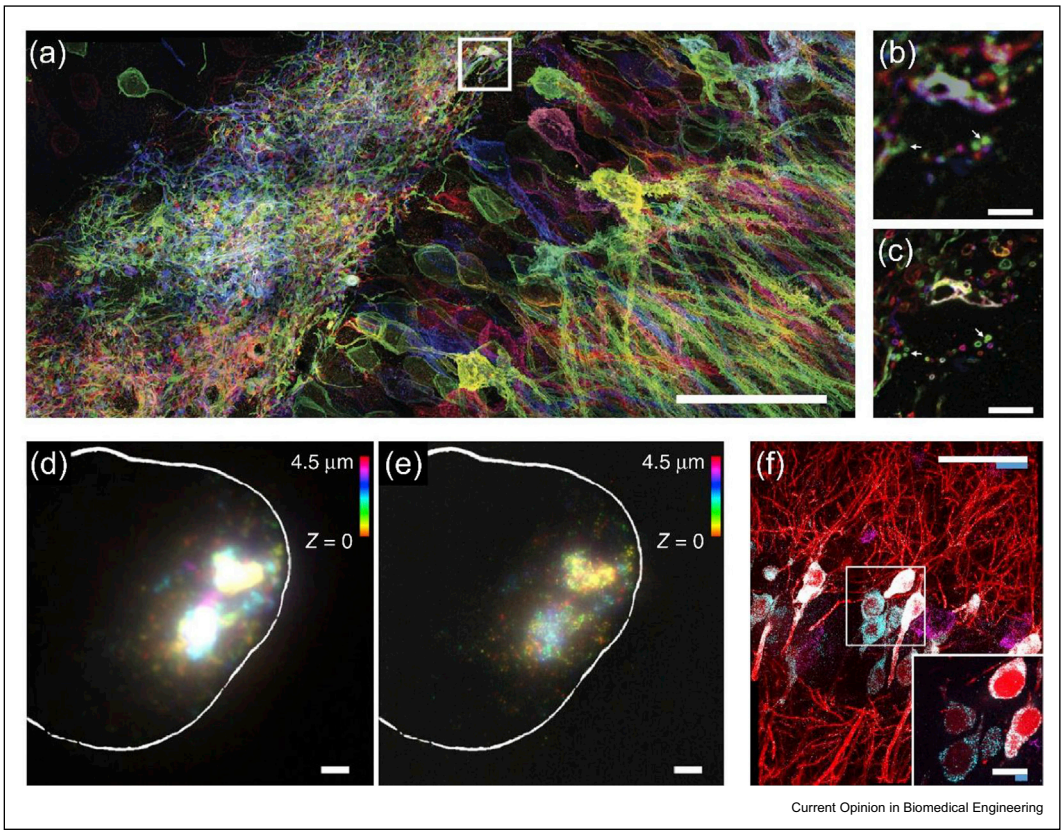


The ExMM concept. (a) Schematic of (*i*) collapsed polyelectrolyte network, showing crosslinker (dot) and polymer chain (line), and (*ii*) expanded network after H_2O dialysis. (b) Photograph of fixed mouse brain slice. (c) Photograph, post-ExM, of the sample (b) under side illumination. (d) Schematic of label that can be anchored to the gel at site of a biomolecule. (e) Schematic of microtubules (green) and polymer network (orange). (f) The label of (d), hybridized to the oligo-bearing secondary antibody top (top gray shape) bound via the primary (bottom gray shape) to microtubules (purple), is incorporated into the gel (orange lines) via the methacryloyl group (orange dot) and remains after proteolysis (dotted lines). Scale bars, (b) and (c) 5 mm. Schematics are not to scale. (g) Confocal fluorescence images of a Thy1-YFP mouse brain slice, stained with presynaptic (anti-Bassoon, blue) and postsynaptic (anti-Homer1, red) markers, in addition to antibody to GFP (green), (g) pre- *versus* (h) post-expansion. Scale bars (g) 2.5 μm; (h) 2.5 μm. Adapted with permission from Ref. [11].

retention ExM (proExM) [13], where proteins, instead of labels, are anchored to the swellable hydrogel network, through the use of a commercially available crosslinker. Antibodies can be delivered to samples post-expansion if a gentle mechanical homogenization technique is used. Alternatively, we demonstrated simple protocols in which fluorescent signals from both genetically engineered fluorescent proteins and exogenously labeled secondary antibodies, directly conjugated to the hydrogel networks, could be well preserved even when subjected to otherwise non-specific proteolytic digestion. For example, a post-proExM confocal image of a slice of mouse hippocampus expressing Brainbow 3.0 (a transgenic labeling strategy that enables stochastic expression of different combinations of fluorescent proteins in the brain, to be revealed by later staining with fluorescent antibodies) clearly indicated the retention of the fluorescent proteins (Figure 2a), which showed much higher resolution than the image obtained pre-expansion (Figure 2c *versus* 2b). As such, proExM is a simple extension of the original ExM method and is already beginning to enable applications in imaging of protein biomarkers in complex biological samples at high resolution, such as the analysis of neural connections and circuits in the mouse brain [19]. In parallel to our efforts developing proExM, two other groups independently published very similar strategies that expand proteins away from each other, enabling either post-expansion antibody staining [20], or selective retention of fluorescent proteins and antibodies that were administered pre-expansion [21] — which both highlights the rapid spread of expansion microscopy, and also suggests that protein-retention forms of expansion microscopy are indeed simple to implement and robust.

Another direction for expansion microscopy is to extend the biomolecules interrogated beyond proteins. In particular, the ability to image nucleic acids at high precision in biological tissues is of tremendous interest for identifying specific microorganism strains and cellular and tissue states in both normal and pathological settings, which could not be achieved using the original form of ExM. We recently developed another strategy that allows for ExM of RNAs [12]. We innovated a small molecule linker that allows RNA molecules to be covalently anchored to the swellable polymer network: one part of the linker is an alkylating reagent that binds

Figure 2



The concepts of proExM and ExFISH/smFISH. (a) Maximum intensity projection of high-resolution confocal microscopy stack following expansion of neurons expressing Brainbow 3.0. (b) Pre-expansion confocal image showing one optical section of the boxed region in f. (c) Post-expansion image of b. Scale bars: (a) 5 μm, (b) 5 μm, (c) 50 μm. Adapted with permission from Ref. [13]. (d) smFISH image of X-inactive specific transcript (XIST) long non-coding RNA (lncRNA) in the nucleus of an HEK293 cell before expansion (white line denotes nuclear envelope in d–e). (e) As in f, using ExFISH. (f) Confocal image of mouse hippocampal tissue, showing single RNA puncta. Inset, one plane of the boxed region (red, YFP protein; cyan, YFP mRNA; magenta, *Gad1* mRNA). Scale bars: (d) 2 μm, (e) 2 μm, (c) 50 μm, inset 10 μm. Adapted with permission from Ref. [12].

guanine, and the other part can link to the polymer network. Thus, this linker can bind RNA (and DNA) to the swellable polymer. Post-expansion, fluorescent *in situ* hybridization (FISH) imaging of RNAs can be subsequently performed with single-molecule precision at high yield, and great specificity, in both cultured mammalian cells (Figure 2e *versus* 2d) and intact brain tissue (Figure 2f). Such post-expansion FISH (ExFISH) methodologies can potentially enable further amplification of signals via techniques such as hybridization chain reaction (HCR), because expanding RNAs away from each other decrowds them and makes room for space-requiring reactions such as HCR; the decrowding of RNAs enabled by expansion microscopy may also help track RNAs over many rounds of hybridization as utilized in multiplexed FISH imaging of dozens to hundreds of transcripts at once [22–26]. ExFISH thus allows for super-resolution imaging of nucleic acids with conventional microscopy in specimens of importance to biology, medicine, and diagnostics. As a testament to the tunability of ExM, a recent variant of ExFISH was published in which polymer-anchorable probes were applied to samples, which were then expanded [27]; the modular, simple architecture of ExM protocols suggests that in addition to a set of universally applicable protocols, a diversity of more specialized protocols can be rapidly developed for use in specific fields of diagnostics.

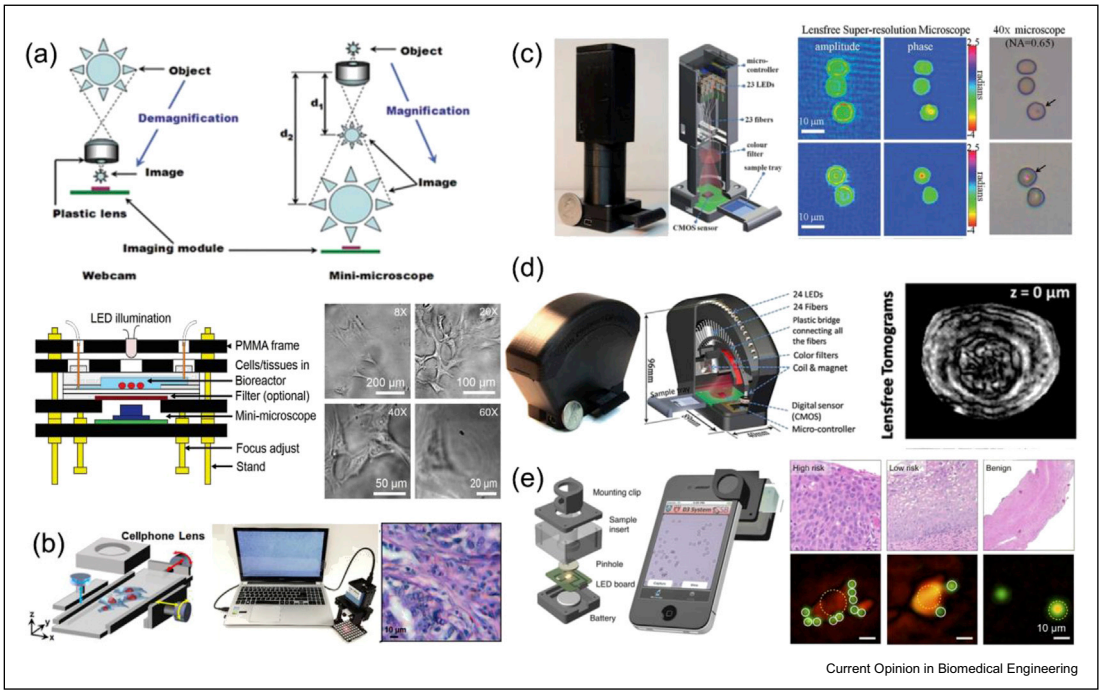
Portable microscopy

Miniature imagers have become useful tools in modern laboratories. They have found many application niches, ranging from the display of physical phenomena to the monitoring of cell behaviors. In particular, many strategies have been devised for the fabrication of mini-microscopes for observing biological samples. For example, Zhu et al. developed a fluorescence microscope encased in a printed box (5.5 × 3.5 × 2.4 cm^3; 28 g), which could easily attach a smartphone as a display, recording, and wireless transmission unit [28]. Schaefer et al. developed an automated and low-cost optical mini-microscope with an autofocusing algorithm [29]. This microscope platform was capable of capturing and

integrating multiple images in a large composed image through image analysis software. We have previously shown that simple inversion of the lens of a webcam created a low-cost, compact microscope ($6 \times 6 \times 4$ cm^3; 40 g) capable of imaging at 20–60× magnifications, which enabled observation of biological samples in real time (Figure 3a) [30,31]. Dong et al. on the other hand, integrated optical microscopy, multi-angle illumination imaging, and a powerful post-processing algorithm for image synthesis in the frequency domain to develop a compact ($8 \times 8 \times 16$ mm^3; 250 g) Fourier ptychographic microscope (FPscope; Figure 3b) [32].

More recently, various POC microscopy strategies with more sophisticated hardware designs and/or algorithms have also been proposed to achieve higher resolutions required to inspect bacteria [33,34] or even sometimes viruses [35] and DNA strands [36,37]. For example, Miller et al. developed a relatively small ($7.5 \times 13 \times 18$ cm^3; 1000 g) and low-cost bright-field/fluorescence "global microscope" for POC applications and validated its use for visual confirmation of the presence of *Mycobacterium tuberculosis* in field-collected clinical sputum samples [38]. Powered by two AA batteries and equipped with LED illumination, this microscope was capable of submicron resolution (0.8 μm) at 1000× magnification. Bishara et al. designed a compact ($5 \times 5 \times 20$ cm^3; less than 100 g), lens-free, on-chip imager capable of digitally collecting and reconstructing holographic images to produce images with a submicron resolution (Figure 3c) [39]. They validated the performance of this device in POC applications by observing malaria parasites (*Plasmodium falciparum*) in blood smear samples. Isikman et al. (Figure 3d) developed a lens-free field-portable tomographic microscope ($96 \times 89 \times 40$ mm^3; 110 g) that obtained holograms and validated the device by observing differently sized microparticles and eggs from the parasitic flatworm *Hymenolepsis nana* [40]. Im et al. developed a digital diffraction-based POC imager that enabled the screening of cancerous and/or precancerous cells and the detection of human papillomavirus (HPV) in cervical samples (Figure 3e) [41]. In addition, Wei et al. designed a mobile phone microscope that was capable of imaging DNA strands in a high-throughput manner using a combination of dark-field imaging, thin-film interference filters, and a computation framework [36]. Single DNA molecules of various lengths were successfully imaged at a sizing accuracy of <1 kilobases. More recently, this setup was further adopted to perform DNA sequencing and *in situ*

Figure 3



Examples of portable microscopes. (a) Mini-microscope fabricated by reversing the lens of a webcam and its use to observe hepatic cancer spheroids. Adapted with permission from Ref. [30] and Ref. [31]. (b) Portable microscope using flipped cellphone lenses and a Fourier ptychographic algorithm to stitch images captured from a sample illuminated by different angles with an LED array and its use in observation of histopathological samples. Reproduced with permission from Ref. [32]. (c) Lens-free portable microscope to obtain holographic images and its use to image cells infected with malaria parasites Reproduced with permission from Ref. [39]. (d) Lens-free portable tomographic microscope and its use to observe an egg of parasitic worm. Reproduced with permission from Ref. [40]. (e) Portable microscope adapted to a smart phone, based on the reconstruction of diffraction patterns and its use to analyze cervical cancer. Reproduced with permission from Ref. [41].

mutation analysis, which enabled accurate identification of pathogens (or specific mutations) at low cost [37].

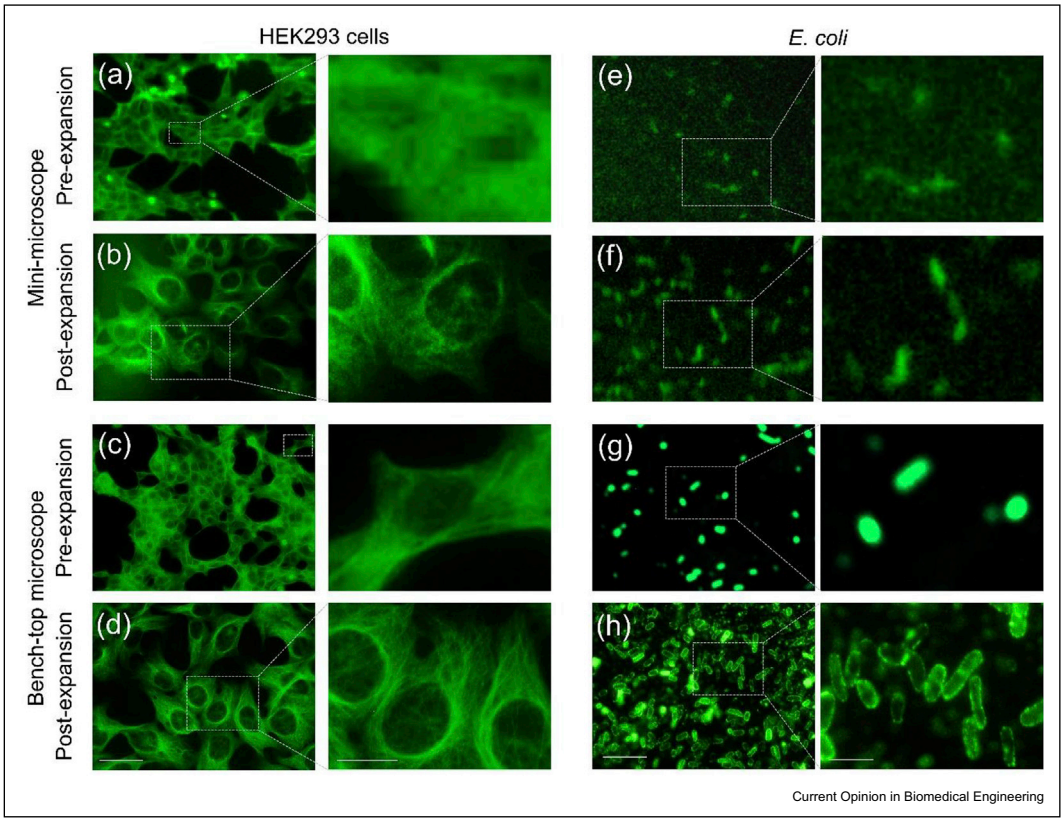
With the success in the use of portable microscopes for POC applications, more frequent integration of the technology with other compatible ones have become prevalent. On the one hand, it is common to integrate the miniature imagers to smartphones [28,42,43] and the recently available wearable devices such as the Google Glass [44]; on the other hand, combination of the mini-microscopy with distinct strategies such as microfluidics for sample preparation and expansion [42], is rapidly emerging.

Expansion mini-microscopy (ExMM)

As aforementioned, while inexpensive mini-microscopy features ultralow cost for extensive use in low-resource settings and remote areas, without additional instrumentation or sophisticated algorithms (including that required in certain cases of portable microscopy), it has hardly been able to achieve a resolution sufficient for POC detection of pathogens at very small scales, such as subcellular structures, single-cell parasites, bacteria, and viruses. An effective and convenient strategy in addressing the insufficient resolution of mini-microscopes without the need for redesigning the hardware would be to adapt the ExM technology — and we recently proved that this strategy we term as ExMM — is feasible [14]: post-ExMM images of HEK293 cells labeled for microtubules showed dramatical improvement in observation of these subcellular structures than those pre-expansion, typical with the low-end optics of the mini-microscopes (Figure 4b *versus* 4a). More importantly, the post-ExMM images were comparable to those captured with a benchtop microscope equipped with high-end optics and camera (Figure 4, c and d), if not better than pre-ExM images (Figure 4c).

In additional preliminary experiments related to POC diagnostics, we labeled a model pathogen *Escherichia coli* (*E. coli*) with a monoclonal antibody that targets lipopolysaccharides on the membrane of the bacteria. The bacteria were then embedded in the hydrogel and expanded approximately 4.5 times in dimension. It was noted that, the presence of *E. coli* could only be imaged using the mini-microscope in post-ExMM samples (Figure 4e), whereas no deterministic signals were detected from the bacteria in their original unexpanded

Figure 4



The concept of ExMM. (a–d) ExMM imaging of tubulin for HEK293 cells. (a, b) Pre- and post-expansion images obtained from mini-microscope. (c, d) Pre- and post-expansion images obtained from bench-top microscope. Scale bars: (left) 100 μm; (right) 50 μm. (e–h) ExMM imaging of *E. coli*. (e, f) Pre- and post-expansion images obtained from mini-microscope. (g, h) Pre- and post-expansion images obtained from bench-top microscope. Scale bars: (left) 5 μm; (right) 2 μm. Adapted with permission from Ref. [14].

state since these bacteria are tiny in size with individual cells measuring about only 0.5 μm (width) by 2 μm (length). The use of polyclonal antibodies further improved the signal-to-noise ratio (Figure 4f). Similarly, the bacterial cells showed much clearer morphology with the bench-top microscope after ExM, as indicated by the well-stained lipopolysaccharides on their membranes (Figure 4h *versus* 4g). It is also estimated that, the chemicals involved in the specimen expansion process can be used at extremely low cost due to the ultralow volume involved in microscopy analysis, down to below ¢ 20 at a volume of <10 μL according to our prior calculations [45]. More importantly, while the chemicals are disposable, the slightly more expensive mini-microscope ($5–100) is not consumable and can be used indefinitely for a large number of assays.

Perspectives

The potential applications of ExMM in POC diagnostics have only been demonstrated in *E. coli* detection at present. Nevertheless, we see broad potential for the role for ExMM in this arena. For instance, in POC diagnostics for malaria, despite the increasing availability of antigen-based rapid tests, microscopy on stained blood smears remains the gold-standard [46]. We envision that ExMM has the potential for widespread utility in POC diagnostics following the further integration of the newly developed proExM and ExFISH and possibly advanced forms of other portable imagers.

ExMM still faces a few immediate challenges, including insufficient physical magnification, signal intensity, and ergonomics. First, the current form of ExM is only capable of a physical magnification of approximately 4.5× [11]. However, small-angle X-ray diffraction determinations suggest that several polymers, similar to the sodium polyacrylate used in the original protocol, exhibit a mesh size of 1–2 nm [47]. Therefore, greater physical expansion factors seem attainable, which would result in higher magnifications and better resolution with minimal instrumentation requirements. Second, due to physical expansion, the density of the labeled fluorophores is inevitably diluted during the expansion process, leading to a reduced signal-to-noise ratio, especially as related to mini-microscopy equipped with both low-end optics and imaging sensors. This issue may be addressed by the HCR expansion of the post-labeling signals [12] or through the use of labels with improved binding capability pre-expansion (*e.g.*, polyclonal *versus* monoclonal antibodies [45]). Finally, current ExM protocols are aimed at scientists, with protocol complexities comparable to immunostaining or *in situ* hybridization, and require many steps, whereas for diagnostics one would want to have potentially a very simple protocol with a small number of steps. We propose the further development of a streamlined ExMM kit that conveniently integrates (a) a disposable device to process all the steps involved in ExM (*i.e.*, labeling, gelation, and expansion); (b) the mini-microscope systems; and (c) the algorithms that enable automated image processing. This integration should simplify the use of ExMM-POC diagnostics for biological specimens of interest by reducing the amount of labor and technical expertise required [45]. With this step forward, we are optimistic regarding the future success of ExMM in providing ultralow-cost and high-resolution imaging for POC diagnostics in resource-limited regions.

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