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Detection of waterborne parasites using field-portable and cost-effective lensfree microscopy†

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Abstract

Protection of human health and well-being through water quality management is an important goal for both the developed and the developing parts of the world. In the meantime, insufficient disinfection techniques still fail to eliminate pathogenic contaminants in freshwater as well as recreational water resources. Therefore, there is a significant need for screening of water quality to prevent waterborne outbreaks and incidents of water-related diseases. Toward this end, here we investigate the use of a field-portable and cost-effective lensfree holographic microscope to image and detect pathogenic protozoan parasites such as *Giardia Lamblia* and *Cryptosporidium Parvum* at low concentration levels. This compact lensless microscope (O. Mudanyali *et al.*, *Lab Chip*, 2010, 10, 1417–1428), weighing ~46 grams, achieves a numerical aperture of ~0.1–0.2 over an imaging field of view that is more than an order of magnitude larger than a typical 10X objective lens, and therefore may provide an important high-throughput analysis tool for combating waterborne diseases especially in resource limited settings.

Water-associated diseases create major problems in the developing parts of the world where there is no reliable infrastructure for proper decontamination of water resources which also brings inadequate access to clean drinking water. The same danger also occurs during natural disasters and wars, where water treatment and sewage facilities lose their functionality. As a matter of fact, the occurrence of waterborne diseases has been dramatically increasing worldwide including in highly industrialized countries such as the United States.^{2–5} Two widely spread examples of such diarrheal diseases include Giardiasis and Cryptosporidiosis which are caused by protozoan parasites, namely *Giardia Lamblia* and *Cryptosporidium Parvum*, respectively.⁶ Unfortunately, these parasites can survive in cold water for several weeks and are resistant to most of the conventional water treatment methods such as chlorination.⁷

Various approaches have been demonstrated so far for identification and quantification of pathogens in drinking and recreational water resources.^{8–25} Traditional culture-based methods are time-consuming and have serious drawbacks due to lack of accurate enumeration methods as well as rapid transition of some of the species into non-culturable state after being released into freshwater.^{11,12}

To address the challenges of these laborious tools, culture-independent techniques have also been demonstrated, which utilize various technologies including polymerase chain reaction

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(PCR),^{13,14} DNA microarrays,^{15–17} surface plasmon resonance,¹⁸ immunomagnetic separation,¹⁹ flow cytometry, as well as optofluidics and others.^{20–26} Although these approaches complement each other and emerge as effective tools for detection of pathogens in water, such platforms require moderately complex maintenance and operation skills together with relatively bulky and expensive architectures which partially hinder their application in field settings.

To provide an alternative solution to this important problem, here we investigate the use of a field-portable lensfree holographic microscope (see Fig. 1) to image and automatically detect pathogenic protozoan parasites such as *Giardia Lamblia* and *Cryptosporidium Parvum* at low concentration levels of <400/mL *without* the use of any pre-concentration steps. As we have demonstrated in our earlier work,¹ this holographic microscope has various important features that make it highly attractive for use in resource limited environments. First, it does not utilize any lenses or other bulky optical components which makes it rather compact as shown in Fig. 1, with a total weight of ~46 grams. This handheld microscope utilizes an incoherent light source (*i.e.*, a simple light emitting diode - LED) to vertically illuminate the samples of interest, which then permits recording of lensfree in-line holograms of the cells/bacteria within the sample volume using an opto-electronic sensor array such as a CMOS chip. The recorded holograms are then digitally transferred to a computer for rapid reconstruction¹ of microscopic images of the sample. This computation does *not* need to be performed locally since wireless transmission of the acquired lensfree holographic images (using *e.g.*, a cellphone) to a central PC station could potentially be used to reduce the hardware and software requirements at the user end. Data load of such a cloud computing model is rather easy to manage since each lensfree holographic image can be compressed (without loss of apparent resolution) to less than 0.1 Mbytes per 1mm² field of view (FOV).¹

Having a total imaging field of view of ~24 mm² (*i.e.*, more than an order of magnitude larger FOV than a typical 10X objective lens) together with a numerical aperture (NA) of ~0.1–0.2, this compact and light weight microscope may provide an important high-throughput analysis tool for combating water-borne diseases especially in resource poor settings. We expect further improvements in these imaging parameters as next generation sensor chips with smaller pixel sizes and larger Mega Pixel counts are made available.

To investigate the performance of our lensfree holographic microscope as a screening tool for water quality management, we imaged heterogeneous and homogeneous solutions of two protozoan pathogens, *Giardia Lamblia* Cyst and *Cryptosporidium Parvum* which are commonly spread by contaminated drinking water (see Fig. 2). In these experiments, parasite suspensions were fixed in 5% Formalin at pH 7.4/0.01% Tween 20 with a concentration of 5×10^6 parasites/mL (Waterborne Inc., USA). Each parasite sample is placed between two identical type-1 glass slides using a micro-pipette.²⁷ This disposable sample is then positioned onto the sample tray located on the side of the lensfree microscope shown in Fig. 1. Fig. 2 left column illustrates the raw lensfree holograms of these pathogens (over selected regions of interest that are digitally cropped from a much larger FOV of ~24 mm²). The middle column of the same figure shows the reconstructed microscopic images of these parasites. The details of this reconstruction process are provided in Ref. ¹. For comparison purposes, the same regions of the sample are also imaged using a 40X objective lens (NA~0.65) as illustrated in the right column of Fig. 2, which very well match to our reconstruction results.

Next, we imaged *Giardia Lamblia* Trophozoite samples using the same lensfree holographic microscope (see Fig. 3). For these experiments, 1 µL of *G. Lamblia* Trophozoite sample (~10⁶/mL) is dropped on the top of a type-0 glass slide and another glass slide is used for

smearing the sample droplet with ~30 degrees spreading angle. Smeared specimen was air-dried for 5 min in room conditions. For the staining process, air-dried samples were sequentially dipped into three jars which contained HEMA 3 fixative solution, HEMA 3 Eosinophilic staining solution (Fisher Scientific), and de-ionized water, which was then repeated 5 times in a row.²⁷

Compared to cyst stage (Fig. 2), Trophozoites have flagella and nuclei, together with a more complicated morphology and sub-cellular structure (Fig. 3). As shown in Fig. 3, holographic reconstruction enables imaging of both the phase and amplitude of these smear samples which nicely match with the 40X objective lens images of the same samples, revealing both the flagella as well as the inner features of the stained Giardia Lamblia Trophozoites.

These images shown in Fig. 2–3 illustrate the success of our lensfree holographic microscope for faithfully imaging Giardia Lamblia Cysts, Cryptosporidium Parvum and Giardia Lamblia Trophozoites. However the concentration of the imaged parasites in these experiments was $\geq 10^6/\text{mL}$, which does not shed any light on the detection sensitivity limits of our platform. As a matter of fact, the lensfree holographic microscope of Fig. 1 has significant advantages to provide rapid screening of large volumes. First, its imaging field of view is $\sim 24 \text{ mm}^2$, which is significantly larger than lens based conventional microscopes. Second, in terms of depth of field, lensfree in-line holography is quite advantageous since it can image a deeper channel, permitting larger volumes to be screened in a single shot.

In order to test the detection limits of our lensless holographic microscope, we further imaged and automatically characterized parasite counts of several diluted samples containing Giardia Lamblia Cysts. We should emphasize that the standard pre-concentration steps^{28–30} that enrich the density of the sample before the detection process are not used here as our aim was to *exclusively quantify the counting limits of our lensfree holographic microscope*.

In these automated parasite counting experiments, we started with an initial concentration of $>1.5 \times 10^6$ parasites/mL, and then diluted it by 1024, 2048, 4096 and 8192 times to create 4 batches of known parasite concentrations, corresponding to 1510/mL, 755/mL, 378/mL, and 189/mL respectively. The original cyst concentration was double checked with a regular bright-field microscope for validating the accuracy of the parasite density. 13 measurements were then made for each one of these dilution levels (*i.e.*, 52 measurements total). In each measurement, the sample solution was placed within a custom-made cuvette that had a controlled depth of 2.5mm. Before imaging, we let the samples in the cuvette sediment for ~20–30 min, after which the cuvette was imaged using the lensfree holographic microscope shown in Fig. 1. These holographic images were then reconstructed to create microscopic images of the sample, which were digitally processed for automated counting of Giardia Lamblia Cysts using a custom developed pattern matching algorithm^{31,32} (for further details refer to the Appendix). The results of this automated counting process are summarized in Fig. 4, which indicates that our lensfree holographic microscope *on average* counted 81.6, 39.9, 23.7 and 16.0 Giardia Lamblia Cysts over the entire imaging FOV ($\sim 24 \text{ mm}^2$) for original cyst concentration levels of 1510/mL, 755/mL, 378/mL, and 189/mL, respectively. Considering the fact that the channel height is 2.5mm in each cuvette, these average count values per FOV correspond to measured parasite densities of 1372/mL, 671/mL, 398/mL, and 270/mL, which exhibit an average error of 9.1%, 11.1%, 5.3%, and 42.9% for each dilution level, respectively (see Fig. 4 for details). In these results, no data points among 52 measurements were eliminated regardless of their value with respect to the mean and the standard deviation. By simply eliminating the maximum and the minimum of the measured values in each batch (which reduces the number of data points to 11 from 13 for each dilution level), then our mean error in concentration measurements reduced to 7.4%, 7.3%,

3.5% and 37.2%, respectively. These results validate the performance of our lensfree holographic microscope to quantify *Giardia Lamblia* Cyst concentration of a solution down to a level of ~380 parasites/mL with a mean error of <10%. For a lower concentration of ~190 parasites/mL, this mean error increased to ~40% which corresponds to a standard deviation of only ~3 parasites per imaging FOV (24 mm²). We should also note that using well established pre-concentration steps such as centrifugation and filtration,^{28–30} we can further improve our detection limit by a factor of *e.g.*, ~100X to claim a detection sensitivity of <5 parasites/mL.

As for the next steps, we aim to test our lensfree holographic microscopy platform in a field setting, where the water samples would normally contain various other particles besides the parasites of interest. Depending on the heterogeneity of the sample solution, modifications of the automated counting algorithm as well as the sample preparation steps may need to be implemented to increase the specificity of each parasite count.

In conclusion, here we have demonstrated lensfree holographic imaging of protozoan parasites, *G. Lamblia* Cyst and *C. Parvum*, as well as *Giardia Lamblia* Trophozoites using a light-weight, cost-effective and field-portable microscope. Furthermore, using the same lensfree holographic unit, we have achieved automated counting of *Giardia Lamblia* Cysts at low concentrations of ~380/ mL with a mean error of <10%. These results demonstrate the promising potential of our field-portable lensfree holographic microscopy platform for combating waterborne diseases especially in resource limited settings. When combined with pre-concentration steps, we should be able to further improve our detection limit to <5 parasites/mL. Towards this end, among other approaches,^{28,29} membrane filtration is a quite promising one that can be applied for specific isolation of bacteria/parasites as described in Ref. ³⁰.

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Appendix

Batch processing and auto-focusing of holographic images for counting of parasites

One of the important tasks when applying lensfree holographic on-chip imaging technology to automated detection and enumeration of micro-objects is to realize batch processing of several holographic images. Therefore it is desired to optimize the digital reconstruction algorithm¹ in order to effectively process multiple images with minimum user interaction. For this end, allocating and deallocating dynamic memory is done in an effective fashion where common batch data such as interpolation grid generated for up-sampling or transfer function generated for digital reconstruction are allocated once throughout the whole process. When a host machine with multiple processing resources (such as GPU clusters) is present, our approach utilizes an automatic batch scheduling for assigning each image to a suitable system in a given resource cluster. The delays when transitioning between images can be eliminated by using minimal inter-system messaging and parallel processing can be maximized by using system and processor dependent threading.

Batch processing also requires refraining from using any experiment-dependent variables within the reconstruction and identification processes. The digital reconstruction algorithm¹ we have utilized includes backward and forward propagation of the optical fields using angular spectrum approach between two planes separated by a vertical distance of z . Therefore, z must be set accurately in order to obtain high quality reconstructions. Even though z is estimated using the prior knowledge about the experimental setup, fine tuning its value within a range of $\pm 20\mu\text{m}$ might be needed for reconstruction of each image. Digital adjustment of this depth parameter in order to maximize the contrast and signal to noise ratio of the output images is done by a custom-developed lensfree *autofocus* algorithm.

For this end, the method we have adopted takes the advantage of Sobel gradient magnitude, which has long been considered as the benchmark in autofocusing.³³ Accordingly, the focus measure (F) of a given image (I) can be expressed as the variance of gradient magnitudes:

$$F(I) = \frac{1}{MN} \sum_m^M \sum_n^N [\nabla S(m, n) - \overline{\nabla S}]^2$$

where the input image I has $M \times N$ pixels and $\overline{\nabla S}$ is the mean of $\nabla S(m, n)$. Gradient magnitudes are calculated for each $I(m, n)$ by using:

$$\nabla S(m, n) = \sqrt{\nabla S_x(m, n)^2 + \nabla S_y(m, n)^2}$$

where ∇S_x and ∇S_y are the horizontal and vertical gradient magnitude matrices obtained by convolving the input image (I) with the following Sobel operators, respectively:

$$S_x = \begin{pmatrix} -1 & 0 & 1 \\ -2 & 0 & 2 \\ -1 & 0 & 1 \end{pmatrix} \quad S_y = \begin{pmatrix} 1 & 2 & 1 \\ 0 & 0 & 0 \\ -1 & -2 & -1 \end{pmatrix}$$

Such a numerical approach ensures that the focus measure (F) reaches its maximum at a vertical distance where the reconstructed amplitude image exhibits well defined objects with the most sharpness and contrast, after which an automated particle detection algorithm^{31,32} can be applied. One example of such characterization for a heterogeneous sample containing Giardia Lamblia Cysts, C. Parvum, 3 and 10 μm beads as well as uncontrolled dust particles is presented in Fig. 5. Using a state of the art GPU, the reconstruction of such an image takes less than one second, and for 1 mm^2 imaging field of view, less than 0.1 Mbytes would be sufficient to be transmitted over the telecommunication network.¹

The same auto-focusing algorithm is also useful to combat a potential tilt of the sample plane. In such tilted samples, the auto-focus function is applied locally in order to bring different parts of the entire field of view into focus; and the particle count of each sub-region, which is separately reconstructed using the optimal local focusing distance, can be combined to obtain the cumulative particle count of the sample.

Fig. 1.

(a) The lensfree holographic microscope¹ used in this study is shown. This fully-functional lensless microscope uses a light emitting diode (at 591nm) and a pinhole of $\sim 50\mu\text{m}$ diameter as shown in (b). The entire assembly weighs ~ 46 grams and achieves a numerical aperture of $\sim 0.1\text{--}0.2$ over an imaging field of view of $\sim 24\text{ mm}^2$, which is more than an order of magnitude larger than the field of view of a typical 10X objective lens.

Fig. 2.

Lensfree holographic imaging results of homogeneous and heterogeneous samples of *G. Lambliia* Cyst and *Cryptosporidium Parvum* are illustrated. The raw holographic images (first column on the left) are acquired using the lensfree microscope shown in Fig. 1; and are digitally processed using our custom developed image reconstruction algorithm.¹ Holographic reconstruction results (middle column) show very good agreement with the corresponding images obtained using a 40X objective-lens (NA~0.65) shown on the third column. Note that because the samples were suspended in a solution, their relative orientations are slightly shifted in the microscope comparison images. Since *Cryptosporidium Parvum* samples are relatively small with weak scattering properties their transmission microscope images are rather faint; therefore we used orange coloured arrows to point to their locations.

Fig. 3.

Lensfree holographic imaging results of *G. Lamblia* Trophozoite smears are illustrated. The raw holograms of the samples are acquired using the lensfree microscope of Fig. 1 and are shown on the left column. The second and third columns at the middle show the reconstructed phase and amplitude images from these lensfree holograms, respectively. The flagella of the *G. Lamblia* Trophozoite samples are clearly visible in both the phase and amplitude images. For comparison purposes the same smear samples are also imaged using a 40X objective lens (NA~0.65) the results of which are shown in the right column, which nicely match to our reconstruction results.

Fig. 4.

Lensfree holographic microscope of Fig. 1 is used to image 4 different batches of diluted water samples containing *Giardia Lamblia* cysts at concentration levels of 1510/mL, 755/mL, 378/mL and 189/mL. For each dilution level, 13 different lensfree holograms corresponding to different samples of the same batch were acquired and digitally processed to reconstruct microscopic images of the samples. These reconstructed images were then automatically counted yielding the number of cysts per frame (over an imaging FOV of ~24 mm²). The above figure illustrates the mean and the standard deviation of these count results, which accordingly measured the average parasite concentration in each dilution level as 1372/mL, 671/mL, 398/mL, and 270/mL respectively. The mean errors in these parasite concentration measurements are 9.1%, 11.1%, 5.3%, and 42.9% respectively. In these results, no data points among 52 measurements were eliminated regardless of their value with respect to the mean. By simply eliminating the maximum and the minimum of the measured values in each measurement batch (which reduces the number of data points to 11 from 13 for each dilution level), our mean error in concentration measurements then reduces to 7.4%, 7.3%, 3.5% and 37.2%, respectively. These results validate the performance of our lensfree holographic microscope to quantify *Giardia Lamblia* cyst concentration of a solution down to a level of ~380 parasites/mL with a mean error of <10%. This also implies that using well established pre-concentration steps such as centrifugation and filtration,^{28–30} we can potentially improve our detection limits by a factor of *e.g.*, ~100X to claim a detection sensitivity of <5 parasites/mL.

Fig. 5.

Automated detection of water-borne parasites (Giardia Lamblia Cyst and *C. Parvum*) within a heterogeneous mixture is demonstrated. This is a reconstructed holographic image captured by the lensfree microscope shown in Fig. 1(a). The digital reconstruction process takes less than one second using a graphics processing unit (GPU). 3 and 10 μm beads as well as uncontrolled dust particles were also present in the same solution. For Giardia samples, the green line points to the automatically detected longer axis of the parasite.