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Microfluidics-based diagnostics of infectious diseases in the developing world

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One of the great challenges in science and engineering today is to develop technologies to improve the health of people in the poorest regions of the world. Here we integrated new procedures for manufacturing, fluid handling and signal detection in microfluidics into a single, easy-to-use point-of-care (POC) assay that faithfully replicates all steps of ELISA, at a lower total material cost. We performed this 'mChip' assay in Rwanda on hundreds of locally collected human samples. The chip had excellent performance in the diagnosis of HIV using only 1 μl of unprocessed whole blood and an ability to simultaneously diagnose HIV and syphilis with sensitivities and specificities that rival those of reference benchtop assays. Unlike most current rapid tests, the mChip test does not require user interpretation of the signal. Overall, we demonstrate an integrated strategy for miniaturizing complex laboratory assays using microfluidics and nanoparticles to enable POC diagnostics and early detection of infectious diseases in remote settings.

Over the past decade, research in microfluidics has produced sophisticated methods for sample processing, fluid handling and signal amplification and detection[1], but many of these individual components have yet to be integrated into a single device[2]. In addition, their traditional reliance on large equipment such as syringe pumps, microscopes and computers for operation, although appropriate for a research or clinical laboratory, has rendered many microfluidic techniques ill suited for remote settings[3,4]. In resource-limited settings such as developing countries, where on-site diagnosis can lead to higher rates of treatment and substantially improve the health of people in disadvantaged settings[5,6], the device must also be low cost. A switch in the strategy by which microfluidics is normally performed will be needed before complex laboratory assays can be miniaturized at low cost for use in remote settings[3,4].

Here we present a new strategy for an integrated microfluidic-based diagnostic device that can perform complex laboratory assays but can do so with such simplicity that it can be performed in the most remote regions of the world. We also present field results on how microfluidics and nanoparticles can be successfully leveraged to produce a functional low-cost diagnostic device in extremely resource-limited settings. We focus on miniaturizing ELISA, which serves as the clinical 'gold standard' for detecting most protein-based biomarkers. ELISA achieves a substantial reduction in background and amplification of signal through serial washings and incubation with reagents. In our assay, called 'mChip' (which stands for mobile microfluidic chip for immunoassay on protein markers), the steps of sample processing and detection mirror those of benchtop ELISA: a heterogeneous immunoassay performed on an easily manufacturable substrate, delivery of multiple reagents and final optical-based detection of signal. Unlike benchtop ELISA assays, however, the mChip can perform these procedures with minimal equipment, can be completed within 20 min and uses only several microliters of blood sample, which can be obtained on site from a needle prick.

RESULTS

Integration of multiple microfluidic technologies

The ability to perform an entire assay at a POC setting requires the ability to integrate multiple microfluidic technologies; integration is increasingly recognized as a central technical challenge in developing functional lab-on-a-chip devices[7]. We have integrated three central microfluidic innovations, each of which is developed specifically for resource-limited settings, on the mChip (**Fig. 1**).

First, we wanted to develop a method of high-throughput manufacturing of microfluidic cassettes at low cost. Traditional manufacturing technologies for microfluidics (such as those for silicon, glass or polydimethylsiloxane (PDMS) materials) are useful for prototyping, but are too expensive and slow for mass production of cassettes. Injection molding is an easily scalable strategy for manufacturing hard-plastic components with features on the order of millimeters and above. It has also been used to produce microfluidic systems, but this process has been challenging because of the large aspect ratios required, difficulty in fabricating a master that can withstand high

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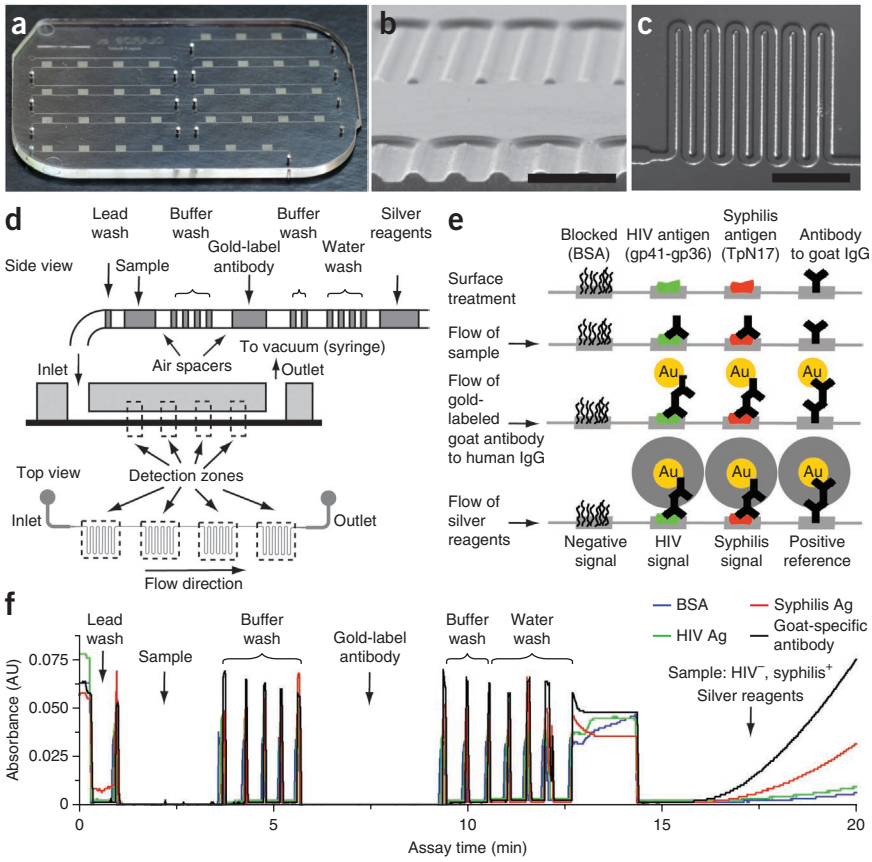

Figure 1 Schematic diagram and pictures of microfluidic device, and data on fluid handling of a POC ELISA-like assay. (**a**) Picture of microfluidic chip. Each chip can accommodate seven samples (one per channel), with molded holes for coupling of reagent-loaded tubes. (**b**) Scanning electron microscope image of a cross-section of microchannels, made of injection-molded plastic. Scale bar, 500 µm. (**c**) Transmitted light micrograph of channel meanders. Scale bar, 1 mm. (**d**) Schematic diagram of passive delivery of multiple reagents, which requires no moving parts on-chip. A preloaded sequence of reagents passes over a series of four detection zones, each characterized by dense meanders coated with capture proteins, before exiting the chip to a disposable syringe used to generate a vacuum for fluid actuation. (**e**) Illustration of biochemical reactions in detection zones at different immunoassay steps. The reduction of silver ions on gold nanoparticle–conjugated antibodies yields signals that can be read with low-cost optics (for quantification) or examined by eye. (**f**) Absorbance traces of a complete HIV-syphilis duplex test as reagent plugs pass through detection zones. High optical density (OD) is observed when air spacers pass through the detection zones, owing to increased refraction of light compared to in the liquid-filled channels. The train of reagents mimics the pipetting of reagents in and out of multiwell plates. This sample was evaluated (correctly against a reference standard) as HIV negative and syphilis positive. Ag, antigen.

temperatures and forces, and stresses experienced by the replica unit that can result in shrinkage and birefringence[8]. Here, by carefully controlling a number of process parameters, we manufactured microfluidic cassettes with spatially precise features, ranging from 1 µm to 1 mm in a single mold, in transparent polystyrene (the same material in multiwell plates for ELISA) and cyclic olefin copolymer (**Fig. 1a–c**). As for lateral flow tests, each cassette costs less than $0.10 in material cost and can be easily produced at a high throughput (1 chip every ~40 s in our process).

Next, we wanted to enable automated delivery of multiple reagents for multistep reactions. In ELISA, multiple additions of reagents and washings ensure high reproducibility and signal-to-noise ratio. In the mChip, we integrated a bubble-based method of reagent delivery (in which metered plugs of reagents are introduced sequentially into a tubing separated by air spacers) to deliver 14 separate reagents (consisting of antibodies, washing solutions and signal-development solutions; **Fig. 1d** and **Supplementary Fig. 1**). This method[9] requires no moving parts[10], electricity or external instrumentation[11] (**Fig. 1a**).

To draw the fluid plugs into the chip, we used a metal spacer to displace 6 ml of air in a syringe to hold a vacuum of 20 kPa during the assay (**Supplementary Fig. 1d**). This method can be performed in the field to deliver fluids with precise volumes and incubation times (coefficient of variation < 5%) that rivals the bulky pipetting robots used in laboratory-based ELISA. Hand-loading of reagents can be performed by local workers at a location and time close to when and where the test will be conducted (**Supplementary Fig. 1c**); one can also load the reagents and prepare the microfluidic cassettes in an automated fashion with the aid of robots (**Supplementary Fig. 1a**,**b**). To our knowledge, the use of passive reagent delivery with stored plugs has not previously been demonstrated to be compatible with whole-blood specimens, which can have a large number of nonspecific interactions. Also, unlike the current study, we have not previously shown this passive delivery method to work smoothly in microchannels that meander with sharp bends.

Finally, we wanted to allow for signal amplification and detection using minimal instrumentation. ELISA achieves a strong signal through enzyme-mediated signal amplification. Rather than forgoing signal amplification, as in most lateral flow assays, we made use of reduction of silver ions onto gold nanoparticles in an immunosandwich[12,13], a procedure that allows a signal to be amplified on a solid substrate under the continuous flow of fluid (**Fig. 1e**). We also used a 'meandering channels' design (**Fig. 1a–c**) to convert a signal developed from nanoparticles inside microchannels to a millimeter-sized scale that can be detected with a wide optical beam without lenses or fine optical alignment[12]. The optical density of the silver film can be measured through low-cost and robust optics such as light-emitting diodes and photodetectors ($0.50 per unit and $6.00 per unit, respectively)[14], which we incorporated into a compact device as inexpensive and simple to use as a cellular phone[15] (**Supplementary Fig. 2**). (By contrast, most microfluidics-based assays require a microscope or bulky sensitive detector to detect low signals generated inside microchannels.) We used this signal-amplification strategy in microfluidics to detect antibody markers to HIV and syphilis (**Fig. 1e**,**f** and **Supplementary Movie 1**). The silver reduction generates sufficient signal within 5 min, with only minimal detectable background development (**Fig. 1f**).

We have previously modeled analyte capture in immunoassays[16] but did not investigate signal enhancement from silver formation, which

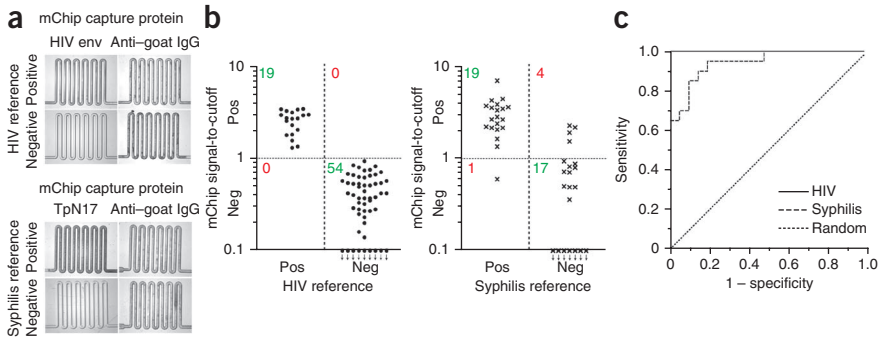

Figure 2 Results of immunoassays performed at Columbia University on commercial specimen panels. (**a**) Images of silver-enhanced signals on detection zones coated with HIV antigen (top group, left column), syphilis antigen (bottom group, left column), and antibodies to goat IgG (anti–goat IgG) (both groups, right columns) as a positive reference. Zones were exposed to positive and negative samples as judged by a reference standard (top and bottom rows, respectively). (**b**) Test results for HIV (left) and syphilis (right) antibodies. Vertical scatter plots of silver absorbance (normalized by cutoff values) for positive (Pos) and negative (Neg) serum or plasma specimens (each human sample is represented by one filled circle for HIV or cross for syphilis). Signal-to-cutoff values smaller than 0.1 are shown at 0.1 (with arrows). See **Supplementary Tables 2–4** for raw data, cutoffs and specimen profiles. (**c**) Receiver-operating characteristic (ROC) curves for HIV (left) and syphilis (right), for illustrating changes in sensitivity and specificity depending on cutoff.

accounts for up to one-third of the assay time. To characterize the influence of gold and time on silver formation, we collected experimental data using a modified assay that controlled the surface density of captured gold-labeled antibodies (**Supplementary Fig. 3**). The kinetics of silver reduction revealed a sigmoid-shaped response, which agrees with observations in related systems[17], and confirmed that considerable signal could be generated in 5 min or less over a range of surface gold density. We used a transport-reaction model, which approximates empirical rates of silver growth to within 10% (**Supplementary Fig. 3**), to guide designs to minimize total assay time.

Development of POC immunoassays

Our test simultaneously detects antibodies against HIV and *Treponema pallidum* (the causative agent of syphilis) from needle-prick sample volumes of blood samples (that is, sera, plasma and whole blood). We chose an HIV-syphilis combination test because HIV and syphilis are treatable in diagnosed pregnant mothers[18], for whom short-course antiretroviral prophylaxis reduces transmission of HIV[19], and treatment with penicillin reduces congenital syphilis[20], which can be fatal for the newborn. Despite the obvious clinical need, a POC HIV-syphilis combination test is not available, resulting in a large number of undiagnosed antenatal-care cases[20]. Our field surveys of more than 60 healthcare workers in three countries (India, Tanzania and Rwanda) confirmed that they would support such a combination test (**Supplementary Table 1**). Detection of host antibodies is a clinically accepted standard for HIV[21] testing and is recommended for syphilis screening in settings where nontreponemal tests (such as the rapid plasma reagin (RPR) test, which is usually performed in a laboratory setting) are unavailable[22].

In the mChip, we used an envelope antigen for capturing HIV-specific antibodies and the 17-kDa outer membrane antigen (TpN17) for capturing treponemal-specific antibodies (**Fig. 2a**) (these antigens produced the best results from 20 antigens screened; other optimization steps such as validation of the optical reader and selection of an appropriate internal positive control are discussed in **Supplementary Figs. 2b** and **4**, respectively).

To investigate the performance of these antigens in a research laboratory, we conducted two parallel sets of assays on validated commercial sera and plasma sample panels, one for HIV (73 samples) and one for syphilis (41 samples). We used the method outlined in **Figure 1**, which requires only a syringe in additional equipment. We used a low-cost, precalibrated handheld reader (**Supplementary Fig. 2**) to measure the optical absorbance for each zone. We differentiated positive and negative samples on the basis of cutoff absorbance values. The test sensitivities, for detection of HIV-specific antibodies and treponemal-specific antibodies, respectively, were 100% (with 95% confidence interval of 98.6–100.0) and 95% (85.4–100.0), with specificities of 100% (99.2–100.0) and 81% (64.2–97.7) (**Fig. 2b**,**c** and **Supplementary Tables 2–4**). The performances of both tests were comparable to those of current commercial ELISA kits (~100% sensitivity and 98–100% specificity for tests detecting HIV-specific antibodies[23] and 82–100% sensitivity and 97–100% specificity for syphilis antibody tests[22]); for syphilis, non-ELISA tests such as RPR and *T. pallidum* hemagglutination (TPHA) serve as laboratory 'gold standards' but cannot currently be miniaturized into POC tests.

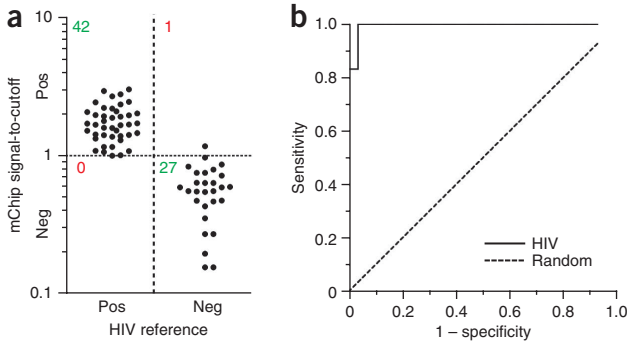

Figure 3 Field results of the HIV immunoassay collected in Muhima Hospital in Rwanda using <1 µl of unprocessed whole-blood sample. (**a**) Signal-to-cutoff ratios for positive (Pos) and negative (Neg) samples. (**b**) ROC curve.

Evaluation of POC immunoassays in Rwanda

Many technologies work well in a laboratory but not in the field, which is characterized by additional complexities not found in a research laboratory such as local specimen strains and subtypes, disease prevalence, real POC infrastructures, environmental conditions and testing protocols[24]. We tested our hypothesis that advanced miniaturization can ultimately simplify a complex assay to such an extent that it can be operated in the most remote regions of the world, where some of the most clinically dire situations exist. In particular, we tested our device in Rwanda (in a collaboration approved by the Rwanda National Ethics Committee), where the HIV prevalence nationally is ~3% and as high as 8% for women in Kigali[25].

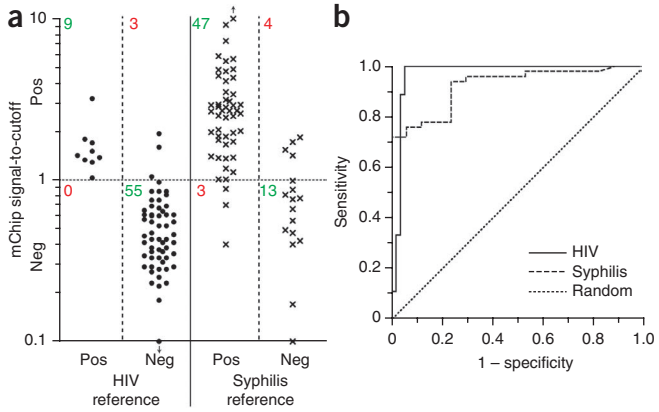

Figure 4 Field results of a HIV and syphilis duplex immunoassay collected in Projet Ubuzima in Rwanda, using 7 µl of plasma or sera. (**a**) Signal-to-cutoff ratios of sera or plasma specimens that are positive (Pos) and negative (Neg) for HIV (circles) and syphilis (crosses). Signal-to-cutoff values >10 are shown at 10, and those <0.1 are shown at 0.1 (both with arrows). (**b**) ROC curves for HIV and syphilis.

First, we tested the device in Muhima Hospital in Kigali, which lacks access to on-site ELISA assays; current turnaround times for ELISA results there are days or weeks because of the need to send the blood samples back to the National Reference Laboratory. We collected whole-blood samples from individuals having recently entered the clinic, and we included roughly equal numbers of positive and negative specimens to ensure reasonable statistical confidence in the final calculated analysis of sensitivity and specificity. In this assay, we used <1 µl of unprocessed whole blood (**Supplementary Movie 2**). (Such a low volume enables collection of blood from various patient types, including infants, for whom even 10 µl is difficult to obtain from a needle prick. Additionally, whereas many ELISA assays can run on whole blood, processing of whole blood in microfluidic devices is often nontrivial due to clogging and coagulation.)

The assay took less than 15 min to complete. Out of a total of 70 specimens with known HIV status (about half male and half female), only one tested false, resulting in overall sensitivity of 100% (98.9–100) and specificity of 96% (88.7–100) (**Fig. 3a**,**b** and **Supplementary Table 5**), rivaling the accuracy of lab-based HIV testing.

We also evaluated in Rwanda the mChip HIV test on 101 archived specimens from couples who received HIV voluntary counseling, many of whom were also positive for hepatitis B, hepatitis C or hepatitis B and C. The results showed a similarly high sensitivity of 100% (98.9–100) and specificity of 94% (88.4–99.7) for our HIV test (**Supplementary Fig. 5** and **Supplementary Table 6**). These experiments verified the ability of our microfluidic device to perform HIV tests on small volumes of whole blood from individuals with a range of clinical statuses and to produce results that require no subjective user interpretation.

We also investigated the ability of mChip to perform a combined HIV-syphilis test. ELISA tests multiple biomarkers in parallel, by testing specimens in separate wells using different reagents. This feature is difficult to achieve in lateral flow assays, in which a common buffer is often inappropriate and nonoptimal for detecting more than one biomarker[26]. Here we ran HIV-syphilis duplex tests at Projet Ubuzima in Rwanda (**Fig. 4**), using samples collected at the clinic for a separate research study on commercial female sex workers in Kigali. There were no criteria for selection other than specimen availability. In the duplex version of the mChip, separate zones on the same microfluidic cassette contained different capture reagents to allow for detection of more than one biomarker. With 67 serum and plasma samples, our duplex test had a sensitivity of 100% (97.8–100) and 94% (87.4–100.0) and a specificity of 95% (89.4–100.0) and 76% (55.7–96.3) for HIV and syphilis, respectively (**Fig. 4a**,**b** and **Supplementary Table 7**). The syphilis test was able to detect treponemal-specific antibodies in samples ranging from weakly reactive (low titer) to strongly reactive (high titer) (**Supplementary Table 7**). Hence, the duplex test as conducted in the field performed similarly to lab-based reference tests[22,23] for these two diseases.

DISCUSSION

Overall, the mChip served as a miniaturized ELISA in resource-limited settings, as it performed equally to lab-based immunoassays, with versatility in the type of blood sample (whole blood, plasma and sera) and in its ability to detect more than one type of marker at once. In particular, the high sensitivities of the mChip for both HIV and syphilis are useful for screening: in remote settings, potentially infected patients can be diagnosed immediately, and their samples can be confirmed by tests with greater specificity, different antigen preparations, test principles and biological targets (for example, cardiolipin-specific antibodies for distinguishing active from past or latent syphilis)[22]. The mChip has advantages over lateral flow assays, the format of most currently used HIV rapid tests[26]. For example, current rapid HIV tests require subjective interpretation of band intensities by the user that can result in false positives in real-world settings[27,28]. Furthermore, the sensitivity of HIV rapid tests has been documented to be lower in sample matrices such as finger-pricked whole blood than for serum, resulting in false-negative tests[29]. Moreover, lateral flow tests cannot easily detect more than one disease simultaneously[30,31], and they are not sufficiently sensitive for detecting many important protein markers[3] (although technological improvements may improve their performance in the future[32]).

By contrast, the mChip quantifies signals using a handheld instrument that is no more expensive or complicated to use than a cell phone, allowing for objective measurements not subject to user interpretation. The mChip retains the most useful features of rapid tests, as it can be operated in the field with no external infrastructure or electricity and with minimal training, with the individual tests costing pennies in material and tens of cents in reagent cost (see **Supplementary Table 8** for head-to-head comparison of mChip, ELISA and rapid test in price, performance, sample volume and other operational characteristics). We accomplished these goals by redesigning microfluidics for resource-poor settings, by replacing conventional microfluidic strategies (including materials, advanced fluid handling, and signal amplification and detection) with low-cost, portable, easy-to-operate materials and techniques.

An ultimate goal of this research is to develop a device for infectious-disease screening of pregnant women located in remote areas to prompt early treatment[33,34]. We analyzed the public health impact of a POC HIV-syphilis combination test (**Supplementary Tables 1** and **9**), using the methods published by the Gates Foundation and RAND[35]. From improving maternal-related and pregnancy-related syphilis outcomes alone, the total disability adjusted life years (DALY) avoided with such a combination test is more than 200,000 in Rwanda (**Supplementary Table 9**). Notably, the cost per DALY avoided in Rwanda is $2.35, well below GDP per capita (**Supplementary Table 9**); hence, this healthcare intervention is cost-effective according to criteria set by the World Health Organization. The cost-effectiveness

metric will be important in translating promising proof-of-concept technologies through the regulatory and distribution hurdles in the developing world (**Supplementary Methods**).

Fluctuations in temperature can potentially affect the performance of our test, most likely through degradation in the wet reagents. Our data show that the most susceptible reagents, wet solutions of gold antibodies and silver-development reagents, were stable for more than 6 months at room temperature (15–25 °C) (**Supplementary Figs. 6** and **7**). This test can also be used outside of the antenatal care clinic, as HIV and syphilis combination testing is important in prescreening of blood donations[31] and epidemiological surveillance (as sexually transmitted infections are particularly poorly monitored in developing countries). Also, this test is relevant for use by primary care physicians and outpatient clinics in developed countries, as on-site diagnosis can lead to higher rates of correct treatment and lower rates of unnecessary overtreatment. Finally, the immunoassay can be extended to other clinically useful markers for sexually transmitted infections such as chlamydia and gonorrhea, for which there are few adequate POC options[20]. Overall, we demonstrate a strategy by which microfluidics and nanoparticles can be integrated to achieve POC diagnosis of clinically relevant infectious diseases in the developing world.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

S.K.S. initiated the study; C.D.C. and S.K.S. designed and conducted the study; C.D.C., T.L., J.W., H.M. and R.R. performed microfluidic immunoassays at Columbia; Y.K.C. developed the compact reader; D.S. and V.L. advised on assay development and provided materials and reagents; H.P. performed computational analysis; L.M. performed reference testing of clinical samples; S.L.B., J.v.d.W., R.S., J.E.J. and W.E.-S. acquired clinical samples and assisted with field studies; C.D.C. and T.L. performed microfluidic immunoassays in Rwanda; C.D.C., T.L. and S.K.S. analyzed data; C.D.C. and S.K.S. wrote the paper; all co-authors edited the paper.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

mChip. The mChip manufacturing process has a throughput of at least 1 per 40 s, with a material cost of about $0.10 per cassette. Features of 1 μm to 1,000 μm can be faithfully replicated. The plastic chip has overall dimensions of 5.4 cm by 8.5 cm by 3 mm. Each channel has dimensions 120 μm width by 50 μm depth by 138 mm length with rounded cross-sections (to reduce formation of tiny bubbles, which can obstruct flow if accumulated) and widens at both ends to a width of 240 μm to through holes of diameter 1.54 mm. Each channel has four meandering sections positioned in series, with each meandering section covering a rectangular area of 6.19 mm^2 (2.75 mm × 2.25 mm) and separated by a distance of 9 mm. We functionalized meandering zones with different capture proteins to create a multiplexed test detecting up to three biomarkers (with the fourth zone as a internal positive control). The clinical need assessment, social impact analysis, visualization of microfluidic chips, surface modification and reagent loading, immunoassay operation, design and validation of compact reader, data acquisition and processing, evaluation of cost and time-to-fabrication of mChip, stability of silver reagents and gold-conjugated antibodies, computational modeling of silver-gold reduction reaction, testing at Columbia University, Rwanda field trials, special considerations for testing devices in developing countries and translation of POC diagnostics to developing countries, are described in the **Supplementary Methods**.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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