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TITLE:  Smartphone Enabled Point-of-care Diagnostics for Operationally Significant Pathogens

PRINCIPAL INVESTIGATOR:  Dr. Ashutosh Chilkoti

CONTRACTING ORGANIZATION:  Duke University
                           Durham, NC  27708-4677

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                Fort Detrick, MD 21702-5012

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## 13. SUPPLEMENTARY NOTES

## 14. ABSTRACT
We developed a novel high-throughput screen in microarray format that identifies optimal pairing of antibody pairs, which led to successful identification of optimal antibody reagents for Ebola having excellent sensitivity. We have identified 24 highly promising clones for antibody reagents used for detecting Salmonella. We successfully developed an approach for "self-calibrating" assay chips that correlate fluorescence intensities to the true known concentration of spiked Ag in serum. We have also identified an optimal strategy for labeling our reagents that is well-suited for our portable assay reader. Furthermore, we have developed 3 redundant designs for our ruggedized, portable reader in case of primary device failure. For assay cassettes, we have devised a simple, low-cost chip that encases all reagents and test components in a user-friendly format. Finally, we devised a development strategy for a smartphone app that automates assay readout.

## 15. SUBJECT TERMS
Nanoscale, nonfouling, polymer brush, inkjet printing, point of care, Ebola, Salmonella, antibody, antigen, smartphone, diagnostics, single-chain variable fragment, phage

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I. INTRODUCTION

Enteric fever is caused predominantly by infection with Salmonella serovars Typhi (Typhoid Fever) or Paratyphi (Paratyphoid Fever). These infections represent a significant global health problem, causing 25 million illnesses and over 200,000 deaths each year. Additionally, at the time this proposal originally was drafted, over 25,000 cases of Ebola virus (EBOV) infection occurred with nearly 11,000 fatalities. For enteric fever EBOV infection, the timely initiation of antibiotic therapy or supportive care (respectively) can significantly decrease morbidity and mortality. Current diagnostic approaches require specialist facilities and trained personnel, and are relatively slow, potentially delaying life-saving therapy. We have developed a point-of-care test (POCT) platform—the “D4 assay”—that uses one drop of blood and requires no mixing of reagents, power, or equipment other than a smartphone. The D4 POCT consists of a nanoscale “nonfouling” — protein and cell resistant— poly(oligoethylene glycol methyl ether methacrylate) (POEGMA) brush coated glass chip that contains two types of printed microspots: “stable” microspots of capture antibodies (cAb) and “soluble” microspots of fluorophore labeled detection antibodies (dAb) (Fig. 1). The D4 assay is so named because it has four steps: first, fingerstick blood is dispensed on the chip, which dissolves the soluble spots of fluorescently labeled detection antibodies (dAbs), which diffuse and bind to their respective analyte-bound capture antibody (cAb) spots and generate a quantifiable optical signal that is detected by a custom fluorescence detector that attaches to a smart phone. The images of fluorescent spots captured by the smartphone camera are converted to analyte concentration by a phone app. The D4 POCT is ideally suited for far forward special operations field settings for the following reasons: it is far more sensitive than existing immunoassays, test cassettes cost pennies and are stable at room temperature for months, and is read with a smartphone, generating data with minimal sample processing and operator training. As described in greater detail in Section III below, the overall goal of this project was to develop a D4 POCT platform capable of early diagnosis of infectious pathogens of operational significance.

II. KEYWORDS

Nanoscale, nonfouling, polymer brush, inkjet printing, point of care, Ebola, Salmonella, antibody, antigen, smartphone, diagnostics, single-chain variable fragment, phage

III. ACCOMPLISHMENTS

A. Major Goals of the Project
The overall goal of this project is to develop a D4 POCT platform for blood markers of infectious diseases for use by special operations’ personnel in any region of the world. Specifically, the proposed work is expected to produce a portable, ruggedized D4 POCT that for timely
identification of infection with Salmonella serovars and Ebola virus (EBOV). This diagnostic is based on the detection of circulating Salmonella and Ebola antigens (Ags) for early and rapid diagnosis of disease. This in turn would allow rapid interventions before reaching critical illness. Specifically, the technical objectives (TOs) are shown below, along with the percentage of completion:

<table>
<thead>
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<th>Description</th>
<th>Completion</th>
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<td>Generate a panel of EBOV-specific capture and detection Abs</td>
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<td>2</td>
<td>Generate a panel of S. enterica-specific capture and detection Abs</td>
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<tr>
<td>3</td>
<td>Ab integration and technology optimization of D4 POCT</td>
<td>50%</td>
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<td>4</td>
<td>Validation of EBOV D4 POCT using blood samples obtained from EBOV-infected primates</td>
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</tr>
<tr>
<td>5</td>
<td>Validation of S. enterica D4 POCT using blood and serum samples from Salmonella-infected patients</td>
<td>10%</td>
</tr>
</tbody>
</table>

**Significant Changes in Methods**

1. Although not part of our original proposal, we modified our procedures to include the conversion of scFv clones to an scFV-Fc format. This markedly accelerated our ability to identify clones that display the optimum binding characteristics. We successfully developed a methodology to perform this conversion in a high-throughput manner.

2. We developed a novel high-throughput screen in microarray format that both identifies paired capture and detection Abs, and identifies those Ab pairs that function with the highest sensitivity. This methodology was applied to identify the best Ab pair for EBOV.

3. For Salmonella, we discovered that flagellin will not be an informative marker for S. Paratyphi. This appears not to be true for S. Thyphi, but we will need to confirm this ourselves. Thus we proceeded to identify numerous highly specific clones for both S. Thyphi flagellin and Vi Ag. These are currently being converted to scFv-Fcs, which will be subjected to the same high-throughput screen used for EBOV. If S. Thyphi Vi Ag works well, we will use it for S. Paratyphi.

**B. Accomplishments Under These Goals**

**Major Activities & Specific Objectives**

Over the past funding year, the major activities of the project revolved around generation of highly-specific assay reagents and optimization of the D4 POCT platform. The specific objectives of the project that were pursued in parallel during the funding period (which correspond to the TOs in the original proposal) were as follows:

1. Generate a panel of EBOV-specific capture and detection Abs
2. Generate a panel of S. enterica-specific capture and detection Abs
3. Ab integration and technology optimization of D4 POCT

**Significant Results & Key Outcomes**

Objective 1: Generate a panel of EBOV-specific capture and detection Abs.

After screening for hundreds of individual scFv clones for binding to sGP in ELISA assays, we ultimately identified several dozen unique sGP-binding clones. From these, we selected 28 unique sGP-binding clones for further analysis. We even identified some that bind well to EBOV, but not Sudan virus, sGP, allowing us to distinguish these two strains. Because of the large number of
sGP-binding scFv clones we identified above, we decided to develop a novel high-throughput screen in microarray format that would: 1) identify paired capture and detection Abs; 2) identify those Ab pairs that detect sGP with the highest sensitivity; and 3) include use of the D4 assay format to ensure that the identified clones will function well in our final assay format. This high-throughput screening approach proved to be very successful, as demonstrated with EBOV (described below).

By applying our screening method, we successfully identified the sGP-binding scFv clone that would clearly function best as a capture Ab (clone A1F3-1). We also identified multiple detection Abs that effectively paired with this capture Ab. These clones are shown below in Fig. 1, and highlighted in green boxes. Importantly, these detection Abs include several that detect both the Zaire and Sudan strains of Ebola virus and one that is specific for the Zaire strain, which will allow us to develop an assay that can distinguish infection by these two strains. In addition, in titration assays, these Ab pairs could detect sGP with a sensitivity of less than 1 ng/ml. The dose-response behavior for each of these Ab pairs in D4 format is shown in Fig. 1, with each pair already showing excellent sensitivity (sub-nanogram/mL) and dynamic range in D4 assay format. We converted the best clones into full length IgGs, which showed improved performance in ELISAs. We are in the process of testing these in the D4 format.

Although not part of our original proposal, we modified our procedures to include the conversion of scFv clones to an scFv-Fc format. This markedly accelerated our ability to identify clones that display the optimum binding characteristics. In support of this change, we have now

Fig. 1: EBOV sGP D4 assay. Results of Ab pair matching in D4 format. Best performing pairs indicated with green boxes.
successfully developed a methodology to perform this conversion in a high-throughput manner. One issue we are dealt with is the exact format in which scFv-Fc constructs are generated. For EBOV, we originally used an IgG1 Fc construct. However, purification of the scFv-Fcs produced proved to be suboptimal. We resolved this issue by changing our expression construct to include an IgG2a Fc segment, which enabled superior purification efficiency.

Objective 2: Generate a panel of S. enterica-specific capture and detection Abs.
We generated Ab pairs specific for S. Paratyphi flagellin, and these were shown to work well in the D4 assay. However, in studies that were not part of our actual proposal, we determined that significant levels of S. Paratyphi flagellin is not detected in the serum of individuals infected with this organism. We thus sought to develop Ab pairs specific for Vi capsular polysaccharide in parallel, and we are currently completing our analysis of both targets target for S. Typhi. To date, we have identified 24 unique flagellin or Vi binding scFv clones for further analysis. The conversion of anti-flagellin clones to the scFV-Fc format has now been completed. These Abs have been slated for testing using the high-throughput Ab pair matching scheme in microarray format described above, which was highly effective for EBOV. We are in the process of converting Vi binding scFv clones to the scFV-Fc format.

Objective 3: Ab integration and technology optimization of D4 POCT.
We have successfully identified the optimal capture and detection spot placement on D4 chips. Chips utilizing a design incorporating capture spots printed within concentric rings of detection reagents showed optimal performance compared to other array layouts. This task was completed ahead of schedule.

In our original proposal we sought to print spots of Ag onto the polymer surface to act as “self-calibrating spots”. Unfortunately, we observed that not all Ags immobilized as well as Abs onto the polymer surface. This is likely due to the diversity in size and structure of different Ags. To address this issue, we pursued 3 parallel strategies for constructing self-calibrating D4 assay chips: (a) printing spots of pre-bound Ab-Ag complexes as a dilution series (b) printing dilution series of anti-dAb Abs and (c) printing printing Ab-Ag chimeras, in which Ags are expressed with linker from Ab scaffolds. These approaches are motivated by the observation that Abs possess the ideal size (~150 kD) and physiochemical characteristics for noncovalent immobilization in the polymer brush surface. Remarkably, each method showed effective immobilization and activity. Based on the results of our experiments—along with considering the cost per assay—we downselected our final approach to strategy (b)—printing anti-dAb Abs as calibration spots (Fig. 2A). We used this approach to correlate fluorescence intensities to the true known concentration of spiked Ag in serum (Fig. 2B). Furthermore, correcting intensities by calibration spots reduces chip-to-chip variation (Fig. 2C).
In our work towards ruggedizing and further miniaturizing the D4 detector, we recognized that the optics were not sufficiently sensitive for visualizing organic fluorophores to the extent needed for our application. To address this, we elected to switch to quantum dot probes (which are several orders of magnitude brighter) as reporters. Quantum dot reporters are not susceptible to bleaching and show favorable optical characteristics (e.g. large Stokes shift between excitation and emissions wavelengths, large quantum efficiency). We pursued 2 main strategies for conjugating dAbs to quantum dot reporters: (1) covalent conjugation by EDC-NHS coupling and (2) coupling of streptavidin-conjugated quantum dots to biotinylated dAb. Based on testing results, we downselected our choice down to strategy (2)—streptavidin-biotin conjugates. Using this approach, we achieve good assay sensitivities in D4 (sub-nanogram/mL) (Fig. 3).

For the portable detector, we have embedded redundancy into our approach by developing 3 detector embodiments. These designs are displayed in Fig. 4, which shows the “handheld”, “flashlight-compatible”, and “pocket” versions of the detector. All 3 models are rugged and portable. This approach was adopted based on SOCOM input, wherein redundancy in readout provides backup plans in case of primary device failure. We continue to iterate cycles of ‘design-test-modify’ for these detector embodiments in parallel with our assay development efforts.

We continue to make progress in developing a D4 with sealed chip architecture. As displayed in Fig. 5, we have devised a simple, low-cost microfluidics chip that is driven to completion by capillary action. The chip encases all reagents and test components in a user-friendly format. Fabrication is also simple—chips and channel features are laser cut directly on a plexiglass sheet, and then adhered directly to a POEGMA-coated glass surface containing assay reagents. Since the chip is capillary-driven, no power, electronics, nor pumps/valves are required for chip operation other than applying liquid to the chip.

Finally, we have initiated development of the smartphone app to quantify spot intensities. This task will be carried out by a team at Duke. Our strategy for software development involves the use of Python code because it is free, open-source, well-documented, and highly versatile. These factors allow for a more agile development cycle and more precise control of our raw data; the versatility of Python’s OpenCV open-source computer vision software package has more intuitive image processing algorithms than our current proprietary software.

C. Opportunities for Training and Professional Development
This project provides an exceptional training opportunity for three individuals – Margaret Lund (research associate), Daria Semeniak (PhD student), and Daniel Joh (MD-PhD student). By working with other senior members of the laboratory, they gained proficiency in a wide range of skills. Examples include polymerization of POEGMA by SI-ATRP, characterization of the POEGMA brush on glass, inkjet printing of the microarrays, assay optimization, and assembly of cassettes/detectors of the “D4” point-of-care test (POCT).

D. Dissemination of Results to Communities of Interest

We made our research accessible and comprehensible to the general community by organizing lab tours for incoming college students at Duke.

E. Plans During Next Reporting Period

In the short-term, we plan to pursue several tasks which are continuations of the 3 Technical (Specific) Objectives outlined above. With respect to Ab development, for Ebola we will first confirm the performance of our anti-sGP IgGs in the D4 format using recombinant sGP spiked into human serum. This assay will then be optimized by varying Ab concentrations and incubation times. For S. Typhi, we are in the process of testing individual Ab clones in the D4 format using recombinant flagellin. Once this is completed, we will subject these Abs to Capture/Detection Ab pairing using our novel high-throughput matching procedure. Optimal pairs will be converted to IgGs and validated in the D4 format using recombinant flagellin. The assay will then be optimized and validated against serum obtained from S. Typhi infected humans. Our anti-S. Typhi Vi Ab clones are currently being converted to scFv-Fc molecules. Once this is accomplished, these Abs will be tested and validated as described for the flagellin Abs above. If the anti-S. Typhi Vi Ab clones prove to function well, we will generate, test, and validate anti-S. Parahyphi Vi Ab clones in a manner used for the anti-S. Typhi Vi Ab clones. As candidates for assay reagents for each pathogen become available and finalized, we will fully characterize assay performance (including matrix effects) to downselect assay reagents that will be used for pre-clinical testing. These assay chips will also be iteratively optimized in conjunction with the 3 detector designs shown in Fig. 4 to ensure compatibility. We are also completing a preliminary algorithm for the smartphone app. The app will be capable of automatic identification of microspot arrays. It will also discriminate between capture spots versus detection spots, and then quantify capture spot intensities for the user. This will all occur in an automated manner.

In the long-term, once final assay reagents are chosen and optimized individually on the chip, we will then combine them in multiplex format. The goal is to complete integration and optimization of the multiplex D4 assay by month 9 of year 2. Months 9-10 will be occupied by testing each assay with simulated blood samples and comparing results to ELISAs produced in-house. The results will be interpreted by an interclass correlation (ICC) between our measure versus ELISA. Our hypothesis is that this sample size will lead to an ICC above 0.8, but the greater utility will be to assess the confidence interval of the ICC, which in samples this size will have bounds of ~+0.10. Bland-Altman methods will assess the degree of bias between the two measures. Next, during

Fig. 5: Self-calibrating chip. (A-B) Sealed, self-clearing, assay chip made from cheap, rugged materials (laser-cut plastic & glass). Images immediately after addition of sample (S: whole serum) and at conclusion of assay. Wash (W) and sample colored with food coloring for ease of visualization. Assay automatically timed and goes to completion after addition of serum, based on capillary action.
Months 11-12 of the funding period, we will test the ability for the D4 POCT to predict group membership (infection vs. no infection) in patient (Salmonella) specimens from Oxford and primate (EBOV) specimens in the BSL-4 facility in Galveston. To interpret the results, we will use logistic regression to assess the degree of segregation measured by area under the curve (AUC). We anticipate that our test will yield an AUC > 0.8.

IV. IMPACT

A. Impact on development of the principal disciplines of the project

If successful, this project will have major clinical impact by introducing an affordable, accurate blood test for early and accurate point-of-care detection of S. enterica and EBOV infection, potentially prior to the onset of symptoms. Such technology has life-saving potential for special operations personnel by allowing clinical interventions before fulminant disease progression.

This project also has potential to make a major scientific impact, as successful demonstration in human and animal cohorts will provide prima facie validation of novel early markers for S. enterica serovars and Ebola virus. This has practice-altering implications since early markers for these diseases are urgently needed.

As a result of this project, we developed a novel high-throughput screen in microarray format that both identifies paired capture and detection Abs, and identifies those Ab pairs that function with the highest sensitivity. This methodology was successfully applied to identify the best Ab pair for EBOV. This approach is broadly applicable to any situation where Ab pair matching is required, and is thus likely to have a major impact specifically in the field of immunoassay development. Our goal is to complete a manuscript summarizing the techniques and our findings in a peer-reviewed journal.

B. Impact on Other Disciplines & Society

D4 platform can target any analyte for which clinical reagents (such as antibodies) are available. Therefore, the approach described in this application is ideal for developing rapid and accurate point-of-care tests for the diagnosis and management of most diseases of operational and global health significance. The endstate is being able to provide an array of D4 cassettes for specific regions of the world, i.e., an array of chips focused on the most important infectious agents on the African continent, or any other particular region of the world where military or global health personnel are deployed. This work also introduces a highly robust, generalized system for the rapid development, validation, translation, and scaling of biomarker tests with potential application to other diseases in need for early markers, in a manner not available to prior researchers in the field. This development would have large impact on military operations and global health applications. Examples include rapid deployment of tests for civilian disaster scenarios, bioterrorism, and population-based validation of novel blood tests for diseases in desperate need of new diagnostic options (e.g. cancer screening).

C. Impact on Technology Transfer

Nothing to report

V. CHANGES & PROBLEMS

A. Changes in Approach and Reasons for Change
Conversion from scFv to scFv-Fc. Although not part of our original proposal, we modified our procedures to include the conversion of scFv clones to an scFv-Fc format. This markedly accelerated our ability to identify clones that display the optimum binding characteristics. We successfully developed a methodology to perform this conversion in a high-throughput manner.

**Salmonella Ags.** For Salmonella, we discovered that flagellin will not be an informative marker for S. Paratyphi B infection. Thus, for S. Typhi, we proceeded to identify numerous highly specific clones for both flagellin and Vi Ag. These are currently being converted to scFv-Fcs, which will be subjected to the same high-throughput screen used for EBOV. If Vi clones function well for S. Typhi, we will use this strategy for S. Paratyphi.

**B. Actual or Anticipated Problems or Delays and Actions to Resolve Them**

**Problem 1:** In studies that were not part of our actual proposal, we determined that significant levels of S. Paratyphi flagellin are not detected in the serum of individuals infected with this organism.

**Solution:** We identified Vi capsular polysaccharide as an alternative target, which we will evaluate once we have completed our analysis of this target for S. Typhi.

**Problem 2:** Our original plan was to directly print calibration gradients of Ags onto POEGMA as internal calibration spots. However, we observed that directly printed protein Ags (typically smaller than Abs) do not always non-covalently immobilize as effectively as printed Abs. This led to some spots of printed Ag to appear quite dim after running the assay because of their poor immobilization.

**Solution:** To overcome this obstacle, we explored 3 candidate approaches, based on the observation that Abs possess the ideal size (~150 kD) and physiochemical characteristics for noncovalent immobilization in the POEGMA brush surface. These approaches were: (1) printing spots of pre-bound Ab-Ag complexes as a dilution series (2) printing dilution series of anti-dAb Abs and (3) printing Ab-Ag chimeras using methods developed in our laboratory, in which Ags are expressed with linker from Ab scaffolds. The results of our experiments allowed us to downselect our final strategy to strategy (2)—printing anti-dAb Abs as calibration spots. We used this approach to correlate fluorescence intensities to the true known concentration of spiked Ag in serum. Furthermore, correcting intensities by calibration spots reduces chip-to-chip variation.

**Problem 3:** In our work towards ruggedizing and further miniaturizing the D4 detector, we recognized that portable optics were not sufficiently sensitive for visualizing spots of organic fluorophores.

**Solution:** We performed parallel experiments using quantum dot probes as reporters instead of organic fluorophores. This approach was chosen since they are several orders of magnitude brighter than organic fluorophores, with much narrower emission wavelengths. Furthermore, quantum dots that emit in the visible range have zero spectral overlap between excitation and emission wavelengths (a large “Stokes shift”), further simplifying the detection optics. We pursued 2 main strategies for conjugating dAbs to quantum dot reporters: (1) covalent conjugation by EDC-NHS coupling and (2) coupling of streptavidin-conjugated quantum dots to biotinylated dAb. Based on testing results, we downselected our choice down to strategy (2)—streptavidin-biotin conjugates. Using this approach, we achieve good assay sensitivities in D4 (sub-nanogram/mL).

**Problem 4:** For EBOV, we used an IgG1 Fc construct. However, purification of the ScFv-Fcs produced proved to be suboptimal.

**Solution:** We are converting our expression construct to include an IgG2a Fc segment which successfully provided superior purification efficiency.

**C. Changes that Had Significant Impact on Expenditures**

Nothing to report
D. Significant Changes in Use or Care of Human Subjects, Vertebrate Animals, Biohazards, and/or Select Agents

Nothing to report

VI. PRODUCTS

A. Publications, conference papers, and presentations

Journal Publications

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<th>Status</th>
<th>Acknowledgement of Support?</th>
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VII. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

A. Individuals on the Project

Name: Ashutosh Chilkoti
Project Role: PI
Research Identifier: 0000-0002-1569-2228 (ORCID ID)
Nearest person month worked: 1
Contribution to project: Contact PI responsible for fiscal and administrative management of the project. Responsible for engineering and assay development efforts, which includes overseeing fabrication and analytical equipment needed to design, build and test the components of the D4

Funding Support:

Name: Margaret Lund
Project Role: Associate in Research
Research Identifier: N/A
Nearest person month worked: 4
Contribution to project: Participated in all aspects of D4 assay fabrication (SI-ATRP of polymer coatings, inkjet printing, cassette assembly, testing/optimization, etc.); assembly and optimization of the detector
Funding Support:

Name: Daria Semeniak
Project Role: Graduate Student
Research Identifier: N/A
Nearest person month worked: 1
Contribution to project: Participated in all aspects of D4 assay fabrication (SI-ATRP of polymer coatings, inkjet printing, cassette assembly, testing/optimization, etc.); assembly and optimization of the detector

Funding Support:

**Name**: Daniel Joh  
**Project Role**: Graduate Student  
**Research Identifier**: N/A  
**Nearest person month worked**: 1

Contribution to project: Participated in all aspects of D4 assay fabrication (SI-ATRP of polymer coatings, inkjet printing, cassette assembly, testing/optimization, etc.); assembly and optimization of the detector

Funding Support: Duke Medical Science Training Program (MSTP) T32 Funding

**Name**: Michael Gunn  
**Project Role**: Co-PI  
**Research Identifier**: 0000-0003-4602-0667 (ORCID ID)  
**Nearest person month worked**: 1

Contribution to project: Responsible for overseeing efforts of generating high-affinity recombinant antibodies for integration into the D4 POCT.

Funding Support:

**Name**: Barbara Lipes  
**Project Role**: Research Associate  
**Research Identifier**: 0000-0003-1195-9463 (ORCID ID)  
**Nearest person month worked**: 2

Contribution to project: Responsible for generating high-affinity recombinant antibodies using single-chain Ab (scFv) phage display technology. Also responsible for animal work required to harvest spleens of immunized mice and eventually screen for high affinity Ag-specific scFv clones based on their binding characteristics

Funding Support:

**B. Changes in Other Support**

**Dr. Ashutosh Chilkoti**

1. **Grants Ended**: North Carolina Biotechnology Center grant—ended 9/30/17; Pfizer grant—ended 9/11/17
2. **New Grants**: USAMRAA SO150005, W81XWH-16-C-0219; NIH, 1R01GM123542; Dr. Reddy’s Laboratories—2 new grants (CIS-Platinate and Paclitaxel); NIH, UG3CA211232; NIH, 1R21CA218940; NSF, 1729671; USAMRMC, DM160400

**Dr. Michael Gunn**  
Nothing to report

**C. Other organizations were involved as partners**

Organization Name: Immucor Inc.
Location of Organization: Norcross, GA
Partner’s contribution to the Project: Collaboration, in-kind support, facilities

Organization Name: Oxford University (Pollard Laboratory)
Location of Organization: United Kingdom
Partner’s contribution to the Project: Collaboration

Organization Name: University of Texas Medical Branch at Galveston (Geisbert Laboratory)
Location of Organization: Galveston, TX
Partner’s Contribution to the Project: Collaboration

VIII. SPECIAL REPORTING REQUIREMENTS

A. Quad Chart
See Section IX (Appendices) below

IX. APPENDICES

Attached as appendices
1. Updated quad chart
2. Copy of journal article which acknowledges support from USSOCOM (Proc Natl Acad Sci U S A. 2017;114(34):E7054 E7062.)
3. Complete summary of Other Support for Dr. Chilkoti
Smartphone POC Diagnostics

Timeline

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Participants
- Sponsor(s): BRAG – USASOC
- PEO: SOF AT&L-ST
- Industry: Duke University

Technology
- Detection of antigens
- Sample to answer, ink-jet printed capture & detection reagents
- Environmentally stable

Transition
- SOF TCCC POR- Medical, & CASEVAC kits

Funding($K)

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The So What
- SOF Medics operating in an austere operational environments need a decision support tool to assist with diagnosing pathogens and infectious diseases
- Diagnostic technology is broadly applicable to target any analyte where antibody pairs are available can be applied

Benefits
- Simple, handheld, battery-operated instrument that is read with a smart phone and analyzes samples obtained from blood
- High sensitivity and specificity equals low false positive rate providing an accurate answer and supporting medical decisions

Updated: 22 Oct 2017
Inkjet-printed point-of-care immunoassay on a nanoscale polymer brush enables subpicomolar detection of analytes in blood

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The ELISA is the mainstay for sensitive and quantitative detection of protein analytes. Despite its utility, ELISA is time-consuming, resource-intensive, and infrastructure-dependent, limiting its availability in resource-limited regions. Here, we describe a self-contained immunoassay platform (the “D4 assay”) that converts the sandwich immunoassay into a point-of-care test (POCT). The D4 assay is fabricated by inkjet printing assay reagents as microarrays on nanoscale polymer brushes on glass chips, so that all reagents are “on-chip,” and these chips show durable storage stability without cold storage. The D4 assay can interrogate multiple analytes from a drop of blood, is compatible with a smartphone detector, and displays analytical figures of merit that are comparable to standard laboratory-based ELISA in whole blood. These attributes of the D4 POCT have the potential to democratize access to high-performance immunoassays in resource-limited settings without sacrificing their performance.

D

diagnostic assessment of protein biomarkers plays an essential role in modern medical practice, and its availability has a considerable impact on clinical evaluation and decision making in human health and disease (1). Diagnostic biomarkers are not only used for identifying disease in individual patients but also for developing treatment strategies, tracking treatment response, monitoring recurrence, conducting clinical trials, and performing epidemiological analysis (2). However, shortages in sophisticated laboratory resources, highly trained personnel, and modern facilities (with clean water, cold storage, and reliable power) have hampered the discovery and use of diagnostic and therapeutic biomarkers in the developing world (3). The mainstay approach for sensitive and quantitative detection of protein biomarkers is the ELISA. In developed countries, highly sensitive ELISA and ELISA-like (4) sandwich immunoassay technologies are readily accessible in centralized facilities and are performed by trained operators or even automated to handle the multistep workflow. Much of the workflow is directed toward reducing biomolecular noise due to nonspecific binding of proteins and other components in complex biological fluids that lower signal-to-noise ratio (SNR). Depending on the assay, steps can include sample preprocessing, liquid transfer, blocking, incubation, and wash steps, in addition to data acquisition and analysis with bulky instrumentation. Taken together, these characteristics represent major barriers to obtaining highly sensitive quantitation of protein biomarkers in limited-resource settings (LRSSs) (3). Furthermore, these barriers can also delay treatment, as test results from laboratory-based immunoassays may not always reach healthcare providers and patients in LRSSs quickly enough to impact critical clinical decisions (1). To address these concerns, we sought to design and implement a broadly applicable protein biomarker detection platform with performance comparable to resource- and personnel-intensive technologies such as ELISA while retaining the many attractive features (i.e., low cost, portability, and ease of use) of “passively” driven platforms such as lateral flow immunoassays (LFIs) (5), paper-based diagnostics (PBDs) (6), and passive microfluidics (PMFs) (7).

In our previous work, we fabricated Ab-based microarrays on nanoscale poly(oligo(ethylene glycol) methacrylate) (POEGMA) polymer brushes that were capable of femtomolar detection of analytes directly from complex biological milieu (8). The Ab arrays were directly spotted onto POEGMA brushes that were grown from glass by surface-initiated atom transfer radical polymerization (SI-ATRP) (8, 9). The POEGMA’s ability to resist nonspecific adsorption of proteins greatly improved SNR by reducing “biomolecular noise,” translating to femtomolar limit of detection (LOD) of protein analytes even from whole blood and serum (8, 10, 11). The observation that Ab microarrays could be directly spotted and noncovalently immobilized onto POEGMA coatings, and that spots of Abs dried and captured within the POEGMA brush retained their activity even after drying and ambient storage, greatly simplified assay fabrication.

Significance

Sensitive quantitation of protein biomarkers plays an important role in modern clinical decision making. This work introduces an inkjet-printed assay platform built on a nonfouling, nanoscale polymer brush, which eliminates nonspecific binding, the largest source of noise in surface-based assays. The assay goes to completion after adding a drop of blood (with no additional reagents or mixing), and the assay can be read with a smartphone-based detector. This technology is significant because it enables high-performance diagnostic testing in blood with minimal infrastructural requirements. Furthermore, its fully printed nature makes it highly customizable and thus broadly applicable to a wide range of diagnostic targets.


Conflict of interest statement: The underlying technology of the D4 was developed by A.M.H. and A.C. and acquired by Immucor Inc. in 2014. This article is a PNAS Direct Submission.

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and subsequent storage of the microarrays. Significantly, the POEGMA-based microarrays also decreased the time needed to perform a sandwich immunoassay by eliminating the need for blocking steps and reducing the number of rinsing steps. Like most other immunoassays, however, this approach still required separate incubation steps for labeling with detection reagents to achieve a quantifiable signal. We therefore sought to build upon this body of work and the robust design concepts of existing “point-of-care test” (POCT) technologies to develop a quantitative and sensitive platform that stores all necessary reagents so that the assay is ready for readout following direct addition of blood or serum, while minimizing user intervention.

Here, we report a quantitative, self-contained, multiplexable immunoassay (the “D4 POCT”) that stores all necessary capture and detection reagents “on-chip” in a dry state, shows durable stability without cold storage, and detects analytes with sensitivity comparable to ELISA directly from whole blood without sample preprocessing.

Results
Design of the D4 Immunoassay. The challenge we faced in redesigning an immunoassay on POEGMA brushes as a POCT, and one that is common to most POCTs, is how to spatiotemporally separate the capture and detection reagents, and how to time the precise sequence of events upon introduction of the analyte into the assay to yield a readout. Whereas recent work in PDAs that use highly innovative and robust sequential delivery techniques has been shown to be effective (12–15), our approach was to instead design a passive POCT that uses lateral diffusion in the polymer brush as the temporally programmable mixing principle. The architecture of the D4 assay consists of a glass chip with a ∼50-nm-thick POEGMA coating that contains two types of microspots that are inkjet-printed on the polymer brush: “stable” spots of capture antibodies (cAb) and “soluble” spots of detection reagents (Fig. 1). Here, the capture and detection reagents are placed in very close proximity to one another (several hundred micrometers), unlike LFIAs in which reagents must travel larger distances. The detection reagent spots are soluble, as they consist of a mixture of fluorescently labeled detection Abs (dAbs) and excipient (such as PEG or trehalose) to enable dissolution upon liquid exposure (Fig. 1 A and B). The D4 assay is so named because of the chain of events that drive the assay to completion upon addition of a drop of blood (Fig. 1 A–D): (A) dispense blood onto chip, (B) dissolution of “soluble” detection reagent spots, (C) diffusion of analyte-bound dAb across surface and binding to respective cAb spots, and (D) detection of binding event by fluorescence imaging. An overall schematic of the fabrication of the D4 assay chip is shown in Fig. 1E and consists of the following sequential steps: (i) SI-ATRP to grow ∼50-nm-thick POEGMA brushes on the glass surface, (ii) followed by noncontact inkjet printing of the cAb from buffer and a fluorescently labeled dAb with a molar excess of an excipient, typically PEG.

D4 Detection of Human IgM/IgG from Fingerstick. We first sought to test the concept of the D4 POCT and chose the detection of human IgG and IgM as a test case, as both analytes are present at high concentration in blood and should thus be detected by the assay. Furthermore, detection of IgG and IgM in the same assay also allowed us to assess the feasibility of carrying out a multiplexed D4 assay. To test this concept, a POEGMA-coated glass slide was stamped with a wax grid to confine the sample to the active area of the chip containing an inkjet-printed Ab array (Fig. 2A). The detection mixture was printed as three outer rings of soluble dAb spots containing a mixture of mouse Cy5-anti-human-IgG and/or mouse Cy5-anti-human-IgM (dAbs with a different epitope against human IgG and IgM than the cAb), with linear PEG (molecular weight 116,000), an excipient that was added to enable dissolution of the dAbs upon contact with blood, and heparin to prevent coagulation of blood. The inner 4 × 4 array contains spots of “stable” cAb. Rows 1 and 4 are an anti-mouse cAb (positive control), row 2 is an anti-human IgG cAb, and row 3 is an anti-human IgM cAb.

Representative steps performed by a user are shown in Fig. 2 B–D. First, a drop of blood from a finger stick (Fig. 2B) is applied directly to the microarray and contained within the hydrophobic corral (Fig. 2C). After a predetermined incubation period (5 min for this experiment), the surface is rinsed with ∼1 mL wash buffer from a squeeze bottle, which displaces the loosely bound blood cells and proteins (Fig. 2D). Interestingly, the blood flows to the margins and binds to the hydrophobic corrals, as seen from the red color around the margins in Fig. 2D, but is completely removed from the nonfouling POEGMA surface. The slide is then imaged with a fluorescence detector. The output of the D4 from a fluorescence scanner is shown in Fig. 2 E–J. Before exposure to blood, the inner 4 × 4 cAb array has no intrinsic fluorescence (Fig. 2E). Negative control experiments in which the D4 chip was incubated with either PBS (Fig. 2F) or whole chicken blood (Fig. 2G) show that only the positive-control spots (rows 1 and 4) generate signal, while middle rows 2 and 3 (specific for human IgG and IgM, respectively) show no fluorescence. Upon incubation with human blood with an array that is printed with a detection mixture of both Cy5-anti-human IgM and Cy5-anti-human IgG dAbs (Fig. 2H), rows 2 and 3 show positive fluorescence signal by “sandwiching” circulating human IgG (row 2) and IgM (row 3) analytes between a cAb and the Cy5-labeled dAb specific to each analyte. In contrast, when the outer dAb spots only contain Cy5-anti-human IgM dAb (Fig. 2I), only row 3 (IgM cAb) is
visible but not row 2 (IgG cAb). The converse is true when the outer spots only contain Cy5-anti-human IgG dAb (Fig. 2).

In summary, this proof-of-concept experiment demonstrated that printing both cAb and dAb together on POEGMA coatings greatly simplifies the sandwich immunoassay by eliminating the multistep procedures typically used in standard ELISA. It also suggested that multiplexed detection should be possible with the D4 assay, a desirable feature that has been historically difficult with LFIAs (16).

Quantitative Single-Analyte D4. The concentration of total Ig is relatively high in whole blood [approximately in the micromolar range (17)], so we next sought to examine the quantitative response of the D4 assay for analytes that occur at lower concentrations in complex biological matrices such as serum and whole blood. Fig. 3A shows representative image data of a D4 assay for the cytokine IL-6 exposed to whole chicken blood with and without spiked analyte (right and left panels, respectively). Similar to the array design in Fig. 2, soluble spots of anti–IL-6 dAb are printed as outer rings surrounding centrally located capture spots of anti–IL-6 cAb. These anti–IL-6 cAb spots were printed alongside spots of vehicle control (PBS) and positive-control capture spots (labeled “ctrl”) comprised of anti-dAb Abs targeting the Fc portion of dAbs. These positive-control spots not only indicate whether dAbs were successfully localized to the active area of the assay but also help quantitatively correct for interassay variation as calibration spots (SI Appendix, Fig. S1). The images in Fig. 3A show minimal background signal, due to the nonfouling polymer brush, near-zero signal from anti–IL-6 cAb spots in the absence of analyte, and brightly fluorescent anti–IL-6 cAb spots in the presence of human IL-6-spiked blood. As expected, D4 signal was absent from vehicle control and brightly fluorescent in positive-control spots in both cases. By measuring the fluorescence emission from cAb spots across a range of different concentrations in IL-6-spiked serum and whole blood (Fig. 3B), we obtained the dose–response curves shown in Fig. 3C and D. For this IL-6–D4 assay, we calculated that the LODs in serum and blood were approximately 6.3 pg/mL and 10.9 pg/mL, which correspond to ~310 and 536 fm, respectively, with a dynamic range spanning greater than three orders of magnitude [details for calculating figures of merit (FOM) are provided in Materials and Methods].

The D4 is a flexible platform for rapid immunoassay development. By inkjet printing spots of stable cAb and soluble dAb for various targets onto POEGMA-coated chips in a layout like that of Fig. 3A we were able to develop D4 chips for a variety of different markers. To demonstrate this versatility, in SI Appendix, Fig. S2 we show the performance of D4 immunoassays pertinent to oncology [prostate-specific antigen (PSA) and alpha-fetoprotein (AFP)], endocrinology (leptin), cytokine profiling (TNFα and IL-6), cardiology [B-type natriuretic peptide (BNP)], and infectious disease (HIV p24), in both serum and whole blood. The quantitative FOM for each analyte are summarized in Table 1. The data show that for each analyte tested the D4 is quantitative and sensitive with LODs that range from approximately 6.3 to 113 pg/mL, dynamic ranges between 2.3 to 3.9 orders of magnitude, and acceptable intra- and interassay coefficients of variation (COV_intra and COV_inter, no greater than 15% and 20%, respectively). While the assays in Table 1 used incubation times of 90 min to maximize sensitivity, we observed that much shorter times can be used for sensitive assay readout. To examine the effect of incubation time on LOD, we carried out a leptin-D4 assay for incubation times ranging from 15 min to 90 min and found that the LOD only increased to 57 pg/mL for a D4 assay carried out for 15 min compared with 38 pg/mL for an assay carried out for 90 min (SI Appendix, Fig. S3).

An important design feature that we also considered in our implementation of the D4 was accounting for variability in factors such as sample volume and dissolution efficiency of soluble dAb, which may impact signal quantitation. Our approach to do so is described in greater detail in SI Appendix. Briefly, however,
Table 1. D4 assay FOM

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<th>Analyte</th>
<th>LOB, pg/mL</th>
<th>LOD, pg/mL</th>
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<th>COV_inter, %</th>
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Pilot Clinical Study with D4. We next sought to test the D4 in human patients using a biomarker that would be useful for POC applications. From the panel of analytes shown in Table 1 we elected to test the performance of a D4 against leptin in a clinical setting. This choice was motivated by a recent study in Uganda by investigators at Duke University and Mulago Hospital which determined that a low serum leptin level (even below 50 pg/mL) is a major biochemical risk factor predicting infant mortality due to malnutrition (18). The authors of this study suggested that leptin measurements could be used to identify and provide targeted treatment to malnourished children at highest risk of death. Unfortunately, many low-resource settings lack the infrastructure to perform hormone assays at such sensitivities and require blood specimens to be transported to larger facilities, which delays potentially life-saving treatment. We therefore speculated that the D4 may provide a useful diagnostic alternative to hormone assays carried out in a centralized laboratory by allowing sensitive and quantitative on-site leptin measurements.
Fig. 5. Pilot clinical studies using D4 to measure leptin levels in pediatric patients. (A–D) Representative D4 imaging of whole blood specimens from an (A) obese patient and (B) lean patient immediately after blood draw. For comparison, D4 data of (C) whole chicken blood (WCB) spiked with 1 ng/mL human leptin (D) and human leptin-negative chicken blood alone are also shown. (E) D4 fluorescence readout compared with results from standard sandwich immunoassay in all patients. D4 data are shown in red and sandwich immunoassay data in black. (Inset) Zoomed graph of lean patients alone (NC = negative control serum). (F) Plot showing correlation between D4 versus sandwich immunoassay (R-squared = 0.965; P < 0.0001, two-tailed t test).

As proof of concept, we conducted a small IRB-approved pilot study at Duke University Medical Center (DUMC) comparing serum leptin levels detected by the D4 assay versus clinical ELISA from 3 lean [body mass index (BMI) <10%] and 10 obese (BMI 83.9–99.58%) pediatric patients. We selected this patient population to clinically test the D4 assay across a wide range of leptin levels, as serum leptin levels are proportional to adipose mass (19, 20). Our pilot studies demonstrated high concordance between D4 and clinical ELISA (Fig. 5). Fig. 5A shows D4 images from a representative obese and lean patient in whole blood, immediately after blood draw. The obese patients exhibited higher fluorescence intensities (indicating higher leptin levels) than lean patients (Fig. 5B), which is consistent with known adipocytokine physiology. Significantly, we found that the leptin levels measured by D4 correlated strongly with those measured by clinical ELISA performed in parallel in a central laboratory at DUMC across all 13 patients (P < 0.0001) (Fig. 5E and F).

The readout of D4 microarray chips shown thus far was obtained using a sensitive table-top fluorescence scanner to assess the sensitivity of the D4 assay. While this approach would allow for POC testing in a peripheral laboratory near or attached to a clinic in LRSs (21), we recognize that a table-top scanner is too burdensome for use in the field. To address this issue, we next investigated the feasibility of portable fluorescence imaging of the D4 assay using a mobile phone-based fluorescence microscope (Fig. 6 and SI Appendix, Fig. S5). This apparatus is a compact and cost-effective imaging system that uses an external lens, in addition to the existing lens of the mobile phone camera (22, 23). The illumination is introduced at an oblique illumination angle of ~75° to increase the SNR of the acquired fluorescence images on the phone.

These experiments imaged leptin-D4 assays in both simulated and patient samples. For comparison, we also imaged the same set of D4 arrays (to maintain consistency) with the table-top scanner in the usual fashion. The fluorescence images in Fig. 6A and B depict representative cAb spots of leptin-D4s (with and without analyte) using the table-top scanner and the mobile phone-based imager, respectively. In both cases, the fluorescence readout behaves as expected for leptin-spiked and leptin-deficient serum (left and right image panels), with good SNR. The dose–response curves using a dilution series of leptin-spiked calf serum are shown in Fig. 6A and B. Both scanner and mobile phone-based imaging modalities showed quantitative, dose-dependent fluorescence intensities. In this experiment, the scanner was more sensitive than the mobile phone, with LODs determined to be 0.037 ng/mL and 0.71 ng/mL, respectively. We next imaged a set of leptin-D4 arrays against clinical specimens obtained from obese patients in our pilot clinical study. The D4 readouts from the scanner (red trace) and from the mobile phone platform (blue trace) are shown in Fig. 6C. In both cases, as expected the data show good correspondence with ELISA values (dashed black trace).

Transitioning to the mobile phone-based detection platform reduced detection sensitivity and interassay consistency, as seen by the higher LOD and larger error bars with the mobile phone device. Nevertheless, these proof-of-concept studies demonstrate the feasibility of merging D4 assay technology with compact, field-portable, cost-effective, and easy-to-use mobile phone-based detection platforms. As mobile phone detector technology, computational imaging, and sensing approaches continue to evolve we expect that the fluorescence collection efficiency and hence sensitivity of these portable, low-cost detectors will rival table-top fluorescence scanners. Despite the lower sensitivity of this first-generation mobile phone detector, the field portability of mobile phone-based imaging provide a detection strategy that is well-matched to complement the robustness of the D4 assay technology with FOM that are sufficient for many clinical applications.

![Image](E7058 | www.pnas.org/cgi/doi/10.1073/pnas.1703200114)

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Storage Stability of D4. Because use of the leptin D4 POCT in LRSs would necessitate long-distance shipment of assay materials to sites around the world, an important issue that needed to be addressed is the need for a “cold chain” and the storage stability of D4 chips. We hence measured the performance of D4 chips targeting leptin at different time points and under elevated temperatures after chip fabrication. Fig. 7 compares dose–response curves of leptin-D4s exposed to analyte-spiked calf serum under different storage conditions. In Fig. 7A, vacuum-sealed packets containing leptin-D4 chips were stored 1–92 d under ambient conditions. The data showed no significant difference in assay performance. Likewise, we also observed minimal difference in assay performance when vacuum-sealed chips were stored at 45 °C for up to 120 h (5 d) (Fig. 7B).

Discussion

The D4 POCT offers a promising platform for democratizing access to sensitive and quantitative assessment of diagnostic protein biomarkers. We first showed proof-of-concept application of multiplexed detection of human immunoglobulins. We observed ELISA-like performance across a wide range of protein analytes when assayed in unmodified serum and blood and showed the feasibility of quantification for multiple analytes from the same chip. Our pilot validation studies showed high concordance between D4 and ELISA in human patients, suggesting that this platform may ultimately be translatable to the clinical setting. Next, we introduced the feasibility of combining D4 chip technology with sensitive and cost-effective mobile phone readout for truly distributed use of the platform. Finally, the D4 chips showed durable storage stability, as evidenced by acceptable assay performance following exposure to heat or prolonged storage.

Much work has been previously done toward developing POCT platforms for LRSs. An “active” approach is to incorporate a fluid handling system where the reagents are stored on the device separately and then mixed and washed using pumps and valves, or alternatively by mechanisms that are conceptually similar but with smaller microfluidic devices (7, 24–27). This pioneering design strategy has seen commercial success in some embodiments (e.g., Abbott i-Stat) and shown encouraging field performance in others (26, 27). However, such systems have many parts, which increases the complexity and cost, and the finite possibility of malfunction from each individual component can limit the robustness of the device.

To date, “passively” driven immunoassays have experienced the most success as POCTs for protein analyte detection. LFIAs—the most widely used embodiment in LRSs—use nitrocellulose strips impregnated with dried capture/detection reagents that are passively colated by applying liquid; this design permits a low-cost, rapid approach to protein detection in a user-friendly manner (fluid application with or without premixing, sometimes followed by buffer addition step and/or signal amplification) (5, 28). Despite its strengths, the LIA is not a substitute for the ELISA for two major reasons. First, LFIAs provide semiquantitative or qualitative readouts (in the absence of a dedicated reader), while conventional ELISA provides quantitative results with high accuracy and precision. Second, most LFIAs are not as sensitive as ELISA (29–31), as more antigen-antibody (Ag–Ab) interactions are necessary to generate a positive signal. This leads to certain clinical scenarios in which LIA technology is unable to meet the required sensitivity to be practically useful (32). Furthermore, PBDs have been groundbreaking in further evolving passively driven devices for use in LRSs and offer many advantages over existing POCTs for their low cost, disposability, and ability to be assembled into multidimensional structures (33, 34). While a frequently encountered shortcoming with PBDs has been limited sensitivity, several innovative signal amplification techniques that reduce detection limit without significantly compromising usability have been described, most notably those using paper networks for automated sequential reagent delivery (12, 15) and enzymatic amplification (35). Such advances have made PBDs a promising platform for “lab-on-a-chip” diagnostics; however, more work remains toward addressing issues such as controlling the consistency of paper matrices, the underlying need for the sequential delivery of reagents and/or washing, challenges with multiplex analysis, and further improving detection sensitivities (34, 36). In contrast, capillary-driven PMFs (e.g., Mbio and Phalanx immunoassay) have overcome challenges in sensitivity faced by LFIAs and PBDs while retaining ease of use, with some platforms reaching subpicomolar LODs within 15 min (7, 37–42). By eliminating “active” elements (e.g., pumps), PMFs reduce the complexity and cost of microfluidic-based designs and significantly reduce instrumentation footprint compared with standard assays. While PMFs show great promise, challenges remain in further reducing instrumentation footprint and/or lowering cost of readers to fulfill the accessibility and sustainability requirements that are critical for successful dissemination and implementation in LRSs.

Our contribution toward democratizing access to clinical diagnostics—the D4—builds upon, but also departs significantly from, the accomplishments of existing “passive” POCT designs such as LFIAs, PBDs, and PMFs. The POEGMA interface is the critical element responsible for the high performance and simplicity of the D4. In the D4, the POEGMA brush is nonfouling in its hydrated state. The hydrated POEGMA brush virtually eliminates nonspecific binding of cells, proteins, and other biomolecular noise (8, 9, 43), yielding high SNRs even at low analyte concentration in complex matrices such as blood and obviates the need for microfluidic separation (preprocessing) of cells.

In the dry state, however, the POEGMA brush readily entraps Abs that are inkjet-printed into the polymer brush. Printing cAbs onto the dry brush without excipient leads to their stable immobilization within the brush and prevents bleeding or dissolution of cAb spots upon hydration with biological matrix (8). This leads to uniform capture spot morphology, which facilitates quantification and analysis. In contrast, printing detection agents with an excess of soluble excipient onto the dry brush creates a reservoir for assay reagents. Contact with blood or other aqueous fluid causes dissolution of the excipient in printed spots of the dAb and releases the dAb from the chip, and the released dAb freely diffuses across the surface and drives the assay to completion. The direct inkjet printing of reagents onto POEGMA brush is another key element of the D4 assay, as it allows precise control over assay geometry, spot concentration, and composition, without the need for covalent coupling steps, which simplifies assay fabrication. Like many existing “passive” designs, the D4 stores all necessary reagents on the same device, but it does so by inkjet printing capture and detection reagents in very close proximity onto a nonfouling polymer brush. This design eliminates the need to control fluid movement with fluids or include sequential liquid transfer steps. Combined, these features

![Fig. 7. Storage stability of the D4. Leptin-D4 chips were stored in vacuum-sealed packets under the conditions described and then run in analyte-spiked calf serum. (A) Dose–response curves for leptin-D4 after 1 d (C) and 92 d (M) of storage in ambient temperature show similar performance. (B) Dose–response curves for leptin-D4 after 24 h of ambient storage versus 120 h (5 d) of storage at 45 °C show similar performance.](https://example.com/figure7.png)
of the D4 platform translate to a robust, self-contained, and quantitive POCT without compromising assay performance or cost.

The low-cost fabrication of the D4 and smartphone-based reader, coupled to its sensitivity and quantitative performance, may offer a scalable and sustainable route to manufacturing multiplexed immunoassays for LRSs that are both affordable and analytically robust. When produced in bulk, we estimate that D4 chips would cost <$1 each (SF Appendix, Table S1) and the 3D-printed mobile phone attachment would cost <$30 each. In terms of workflow, we envision that the D4 platform can provide a streamlined protocol for protein analyze detection in both healthcare and research settings and can expand the capabilities of both. The ability to achieve multiplexed results with ELISA-like sensitivity after a single incubation/rinse step and without blocking greatly simplifies protein quantification, reduces time to results, and ultimately lowers costs. Admittedly, creating a multiplexed test in LFIA format is also relatively straightforward as it involves spotting multiple capture reagents onto a strip or adding additional test “lines.” However, the LFIA is highly dynamic, as its constituents are constantly changing at a given location across a test strip and never fully equilibrate. Subsequently, assay readout relies on binding kinetics specifically during which reactants are sufficiently close to interact on a molecular level, and this varies for each individual test. Unless each test is isolated from the others, the same assay condition (e.g., running buffer, porous membrane, and flow rate) is applied to all tests, and one set of conditions is often not optimal for the multiple analytes of interest. While isolation of individual tests is not overly difficult and various strategies to this end are available (44), this adds to complexity of sample handling and cost. In contrast, the design of the D4 assay—by effectively colocalizing the capture and detection reagents and relying on 2D diffusion and equilibration “above” a nonfouling polymer brush—allows multiplexing without significantly increasing the assay complexity.

Difficulty in storage is a major factor restricting the implementation of laboratory-based tests in LRSs. We speculate that the storage stability of D4 is related to the high oligo(ethylene glycol) density of the POEGMA “bottle brush,” which provide a protective environment that stabilizes the printed proteins against denaturation (8, 45). To put our results into context, LFIAAs are already well-suited for LRSs since they are generally stable for 1 to 2 y at ambient temperature and 6 mo at elevated temperature (45 °C) (16), and stability has also been shown in other POCTs (46, 47). The timescales for storage stability used in our studies with the D4 (Fig. 7) were shorter than those determined for LFIAAs. Thus, while encouraging, we recognize that further studies are required to characterize the storage limitations of the D4 and, if needed, modifying packing and/or printing solutions with existing methods for reagent stabilization (35, 48) to meet the storage demands of LRSs.

For patient care, we envision that this technology will provide a robust POCT that is widely applicable for the diagnosis of a disease by quantification of the levels of one or more markers for which orthogonal Ab pairs are available. In the developed world, this will bring highly sensitive immunoassays to first responders and to the bedside, while in LRSs it will allow them to simply become more routinely available. The low cost of the chips and detectors also suggests that wide dissemination of this technology is feasible, so that it would also provide new opportunities for spatiotemporal mapping and remote analysis of measurement results, which would be highly valuable for epidemiology in general. However, it is worthwhile to note that the actual need for ELISA-like sensitivities depends on the disease and biomarker, which must be considered when deciding between the D4 POCT and other robust alternatives such as LFIAAs or PBDs when such sensitivities are not required.

There are several challenges that still need to be resolved. The first is the requirement to remove the blood from the chip before imaging. Because proteins do not adsorb to the POEGMA brush surface and cells similarly do not adhere and are only settled on to the surface by gravity, removal of the blood from the chip is currently easy to perform using a buffer rinse. We acknowledge, however, that the open wash requirement is not ideal for biosafety reasons and possible specimen contamination. The ability to read out the assay in a sealed system with an automated wash or even in the presence of blood would make this a completely hands-off POCT, which is desirable. We are currently pursuing several solutions to this problem, including a PMF-based enclosed chip design that passively removes the blood before readout, as well as electronic modes of detection where the blood does not interfere with the signal, which will be reported separately. The second issue is the sensitivity of the fluorescence detector. While the current design of the cell phone detector is sensitive enough for many clinical applications, we believe that optimization of the detector design will yield the necessary 10- to 20-times improvement to match the performance of table-top fluorescence scanners that are the “gold standard” for imaging microarrays. This assessment is supported by reports of mobile phone-based fluorescence imaging of individual fluorescent nanoparticles and viruses (22) and fluorescently labeled single DNA molecules (23).

Third, while the ubiquitous nature of mobile phones makes it a promising platform for dedicated and nondedicated POCT platforms, there will be challenges with effectively integrating them into healthcare systems. A thorough discussion of this topic is provided in the review by Byrnes et al. (23), but in brief, issues related to developing protocols for transmitting and interpreting results and subsequently making management decisions from POCT data must be resolved. Furthermore, the diversity of phone models and short life cycles in both hardware and software lead to challenges in compatibility and maintaining common standards; the slow pace of regulatory approval also introduces its own set of obstacles. However, these are challenges faced by the entire field of mobile phone-based diagnostics and are not limited to the D4 POCT. We believe the widespread dissemination of phones, their user friendliness, and their computing power make them very attractive vehicles toward democratizing access to gold-standard diagnostic testing. In conclusion, the D4 POCT, combined with the field portability of mobile phone-based imaging, may pave the way for high-performance and user-friendly diagnostic immunoassays that are virtually independent from infrastructural requirements.

Materials and Methods

SI-ATRP of POEGMA on SiO\textsubscript{2} Substrates. In brief, POEGMA surfaces were deposited by SI-ATRP, specifically using an activator regenerated by electron transfer approach under aqueous conditions (49, 50). The experimental conditions used to create POEGMA polymer brush coatings “grafted from” substrate surfaces for our experiment are described in extensive detail elsewhere (8). These methods involve first functionalizing the substrate surface with a bromide ATRP initiator and then immersing the substrates in polymerization solution, producing surface-tethered brushes of POEGMA ~50 nm thick.

Spotting of Antibody Microarrays. All capture/detection Ab pairs and Ag were obtained from R&D Systems. The dAbs were directly conjugated to fluorescentophores per the manufacturer’s instructions. The cAbs (1 mg/mL) were spotted onto POEGMA-coated substrates using a PerkinElmer Piezorray noncontact printer under ambient conditions at 1 mg/mL concentration. Spots of soluble detection reagents were composed of dAbs (1 mg/mL) mixed with excipient (1 mg/mL PEG 115,000 or 0.25 mg/mL trehalose) and printed in a similar fashion. For experiments using fingerstick blood (Fig. 2), spots of heparin were also included in arrays to maintain anticoagulation. After printing, D4 chips were placed under vacuum desiccation (30 KPa) overnight to facilitate noncovalent immobilization of Abs into the polymer brush.

D4 Immunoassay. To generate dose-response curves, D4 chips were incubated with dilution series of analyte-spiked calf serum (Clontech) or whole chicken blood (Innovative Research, Inc.) for 90 min. Substrates were then
briefly rinsed in 0.1% Tween-20PBS then dried. Arrays were imaged on an Axon Genepix 4400a tabletop scanner (Molecular Devices, LLC). The limit-of-blank (LOB) was estimated from the mean fluorescence intensity ($\mu$) and SD ($\sigma$) from 10 blank samples, defined as LOB = $\mu_{blanks} + 1.645\sigma_{blanks}$, as described by Armbruster and Pry (51). LOD was estimated from spiked low-concentration samples (LCS) above the LOB, such that LOD = LOB + 1.645$\sigma_{LOB}$. Low, intermediate, and high concentrations within the working range of each assay were used to determine the COV intra and COV inter. The dynamic range (DR) was defined as the range of concentrations from the LOB to the greatest concentration that had a fluorescence signal greater than 3$\sigma$ of that from the next-lower concentration in the dilutions series. Data were fit to a five-parameter logistic (5-PL) fit curve (52) using OriginPro 9.0 (OriginLab Corp.). Details on the effect of shorter incubation times (15 min and 60 min) on assay results are shown in SI Appendix, Fig. S2.

D4 and ELISA Measurements of Patient Leptin. Approval for clinical studies was obtained from the Duke University Health System Institutional Review Board (protocol no. Pro00064838). Parental assent and subject assent were first obtained, and then 0.5–1.0 mL of blood was drawn in a lavender-top collection tube containing K2-EDTA. For D4 assays in whole blood the blood was directly applied to D4 chips immediately after collection then processed and analyzed as usual. For D4-sandwich immunoassay comparisons the tube was then centrifuged for 10 min at 1,500 × $g$, and the resulting serum was aliquoted into cryovials and stored at −20 °C for later use. Samples were then thawed and analyzed with D4 and ELISA in parallel. Standard leptin sandwich immunoassays (Quantikine ELISA kit; R&D Systems) were performed per the manufacturer’s instructions, which required a 100x dilution with assay diluent buffer for all specimens. For D4 measurements, specimens from lean patients were directly run without dilution. In contrast, we found that obese patient specimens had extremely high leptin levels, above the upper limit of quantitation of the leptin D4. Thus, all specimens from obese patients (to maintain consistency) were diluted 100-fold. Subsequent analysis was performed on a desktop computer for this study and can be conducted by a custom-developed smart application running on the same phone in future designs.

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9. Ma H, Li D, Sheng X, Zhao B, Chilkoti A (2006) Protein-resistant polymer coatings on the upper limit of quantitation of the leptin D4. Thus, all specimens from obese patients (to maintain consistency) were diluted 100-fold. In contrast, we found that obese patient specimens had extremely high leptin levels, above the upper limit of quantitation of the leptin D4. Thus, all specimens from obese patients (to maintain consistency) were diluted 10x with whole calf serum (and not sandwich immunoassay diluent buffer, to effectively maintain complexity of the biological milieu) before running the leptin D4.

Mobile-Phone Imaging of D4 Arrays. The mobile phone-based D4 assay reader device was created by integrating a 3D-printed optomechanical attachment to a smartphone (Lumia 1020; Nokia) which included an oblique illumination source for highly sensitive fluorescence detection (SI Appendix, Fig. S5) (22, 23). A standard microscope slide can be inserted into the smartphone attachment from the side and illuminated by an excitation beam from a battery-powered laser diode (638 nm, 180 mW; Mitsubishi Electric) at an incidence of ~75° from the back side (SI Appendix, Fig. S5A). The fluorescence signal of the sample was collected from the other side of the glass slide by an external lens ($f_2 = 2.6$ mm; UCTronics) and passed through an emission filter (690/50 nm; Semrock) before entering the smartphone camera unit (SI Appendix, Fig. S5A). The smartphone camera is equipped with a 2/3-inch complementary metal-oxide semiconductor sensor with 41 megapixels and 1.72 μm in pixel pitch. The default smartphone camera lens assembly (Carl Zeiss) has an effective focal length of $f_1 = 6.86$ mm, and therefore the nominal magnification (M) of the smartphone-based fluorescence microscope is $M = 0.26 x$. This mobile microscope design provides a high-speed resolution of ~0.98 μm over an imaging field of view of ~0.8 mm2 as characterized earlier (53). The size of the device is about 14.5 x 8.0 x 7.8 cm in length, width, and height, respectively, and the weight of the attachment excluding the smartphone is ~185 g. For a typical fluorescence image, an integration time of 4 s was used on the mobile phone. Fluorescence images were initially saved in raw DNG format (7,152 x 5,368 pixels) on the phone memory and subsequently converted into 16-bit TIFF for further analysis. Image analysis and quantification were performed by ImageJ on a desktop computer for this study and can be conducted by a custom-developed smart application running on the same phone in future designs.

**OTHER SUPPORT**

**CHILKOTI, ASHUTOSH, Ph.D.**

**Changes:**

- **Grants Ended:** North Carolina Biotechnology Center grant—ended 9/30/17; Pfizer grant—ended 9/11/17
- **New Grants:** USAMRAA SO150005, W81XWH-16-C-0219; NIH, 1R01GM123542; Dr. Reddy’s Laboratories—2 new grants (CIS-Platinate and Paclitaxel); NIH, UG3CA211232; NIH, 1R21CA218940; NSF, 1729671; USAMRMC, DM160400

**Active**

**Title:** Center for Environmental Implications for Nanotechnology (PI – Wiesner)

**Supporting Agency:** National Science Foundation NSF-EF-0830093

**Time Commitment:** 0 Academic/0.2 Summer (~1.6% effort)

**Performance Period:** 9/15/03 – 8/31/18

**Level of Funding:** $20,890 (Annual Direct)

**Grant Officer:** Irwin Forseth, iforseth@nsf.gov, 703-292-7862

**Goals and Specific Aims:** The proposed Center explores the role of nano-scale phases in ecosystems, their interactions with organisms, and the mechanisms by which they exert their influence. The Center will translate this knowledge into the language of risk assessment /to provide guidance in interpreting the role of anthropogenic sources of nanomaterials and their relative impacts.

**Title:** Elastin Fusion Proteins

**Supporting Agency:** NIH/NIGMS 2R01 GM061232

**Time Commitment:** 1 Academic (~8.3% effort)

**Performance Period:** 4/1/00 – 6/30/18

**Level of Funding:** $209,349 (Annual Direct)

**Grant Officer:** Dina Wilson, dina.wilson@nih.gov, 301-594-9251

**Goals and Specific Aims:** The overall objective of this competing R01 application is to discover new peptide motifs that seamlessly encode both lower critical solution temperature (LCST) phase behavior and bioactivity, and to use these new sequences to create the “next-generation” of LCST polymers with intrinsic biological activity and self-assembled structures with morphological diversity and enhanced stability.

**Specific Aim 1:** Characterize the sequence space of Pro- and Gly-containing motifs that polymerize into LCST peptide polymers.

**Specific Aim 2:** Investigate the self-assembly of LCST block copolymers that exhibit hysteretic phase behavior into kinetically stabilized nano-mesoscale structures.

**Specific Aim 3:** Create LCST peptide polymers with intrinsic biological function.

**Specific Aim 3:** Carry out in vivo efficacy studies of a subset of PODs from SA2 to determine the long-term therapeutic potential of GLP-1 PODs.

**Title:** Collaborative Research: Supramolecular Materials by Nucleic Acid Block Copolymer Self-Assembly

**Supporting Agency:** National Science Foundation DMR-14-11126

**Time Commitment:** .05 Summer (~0.4% effort)

**Performance Period:** 7/15/14 – 6/30/18

**Level of Funding:** $390,000 (Total Project Cost)

**Grant Officer:** Elizabeth Gebremendhin, (703) 292-4444

**Goals and Specific Aims:** The overall goal of this interdisciplinary, collaborative research proposal is to design and synthesize polynucleotide (co)polymers with chemical functionalities that drive solution self-assembly into
micelles and that harness DNA’s molecular recognition properties to create oriented, supramolecular polynucleotide networks over large areas on surfaces.

**Title:** Treating Melanoma with a Molecularly Engineered, Long Circulating Immunotoxin  
**Supporting Agency:** NIH 1R21-EB019041  
**Time Commitment:** 0.24 Academic (~2% effort)  
**Performance Period:** 9/15/2015 – 6/30/2018  
**Level of Funding:** $125,000 (Annual Direct)  
Grants Officer: Angelos Bacas, ab329b@nih.gov, 301-451-4785  
**Goals and Specific Aims:** The proposed research will develop a new bioengineered drug by InStealth polymer conjugation, in which a polymer that has long in vivo circulation and good tumor accumulation will be directly grown from the N- or C-terminus of a —biologic—drug—a protein that consists of an antibody fragment that homes in on melanoma cells, and which is fused to a potent bacterial toxin that kills cells. We will demonstrate the utility of the InStealth™ technology in improving the treatment of melanoma by this polymer modified protein drug.

**Title:** Non-immunogenic PEG conjugates of Biologics  
**Supporting Agency:** North Carolina Biotechnology Center  
**Time Commitment:** 0.14 Academic (~1.2% effort)  
**Performance Period:** 09/01/2016 - 02/28/2018  
**Level of Funding:** $100,000  
Grants Officer: Joan Pifer, joan_pifer@ncbiotech.org  
**Goals and Specific Aims:** The outcome of the project will be the identification of a fully optimized exendin-POEMGA conjugate with minimal antigenicity, and a technology that provides a superior alternative to PEGylation, with application to the entire class of biologic drugs.

**Title:** SO150005: Smartphone Enabled Point-of-care Diagnostics for Operationally Significant Pathogens  
**Supporting Agency:** USAMRAA, W81XWH-16-C-0219  
**Time Commitment:** 0.9 academic months (~7.5% effort)  
**Performance Period:** 9/28/16-9/27/18  
**Level of Funding:** $343,575, total funding to date  
Grants Officer: Benjamin Chitty, Benjamin.chitty@socom.mil, 813-826-5162  
**Goals and Specific Aims:** The overarching goal of this project is to develop point-of-care tests (POCTs) against infectious diseases of operational significance, ready for large-scale validation against human samples.

**Title:** Magnetically directed single cell transcriptome analysis  
**Supporting Agency:** NIH, 1R01GM123542  
**Time Commitment:** 0.5 summer months (~4.2% effort)  
**Performance Period:** 9/20/16-8/31/21  
**Level of Funding:** $28,973 (Annual Direct, Chilkoti portion only)  
Grants Officer: Jennifer Billington, billingj@nigms.nih.gov, (301) 594-5243  
**Goals and Specific Aims:** Dr. Chilkoti will oversee the development of the immunoarray’s POEGMA surface during the early years of the assay development. He will work closely with Dr. Yellen in optimizing the overall design of the chip to accomplish this goal and directly supervise staff during all steps of the process.

**Title:** Self-assembled microporous networks for cell culture  
**Supporting Agency:** EMD Millipore  
**Time Commitment:** 0.9 Academic months (~7.5% effort)  
**Performance Period:** 5/1/17-4/30/19  
**Level of Funding:** $200,000 (Annual Direct Costs)  
Grants Officer: Martin Zillman, martinzillmann@emdmillipore.com
**Goals and Specific Aims:** Work on developing POP microcarriers will proceed on a two year, two-phase timeline. Phase one will be devoted to developing a reproducible microcarrier prototype capable of supporting cell growth and rapid dissolution for cell recovery.

**Title:** Delivery of paclitaxel by Recombinant Polypeptide Nanoparticles

**Supporting Agency:** Dr. Reddy’s Laboratories, Ltd.

**Time Commitment:** 0.75 Academic months (~6.2% effort)

**Performance Period:** 3/30/17-3/30/18

**Level of Funding:** $393,000 (Total Project Costs)

**Grants Officer:** Ajaya Reka, areka@drreddys.com

**Goals and Specific Aims:** Our second goal is to design and test a “next-generation” nanoparticle that binds endogenous albumin, and thereby enhances the pharmacokinetics, biodistribution and tumor targeting ability of the ELP-PTX nanoparticle.

**Title:** Delivery of Cis-Platinate by Recombinant Polypeptide Nanoparticles

**Supporting Agency:** Dr. Reddy’s Laboratories, Ltd.

**Time Commitment:** 0.75 Academic months (~6.2% effort)

**Performance Period:** 3/30/17-3/30/18

**Level of Funding:** $159,000 (Total Project Costs)

**Grants Officer:** Ajaya Reka, areka@drreddys.com

**Goals and Specific Aims:** The objective of this project is to develop two alternative methodologies to create cis-platinate loaded nanoparticles of a recombinant elastin-like polypeptide (ELP)

**Title:** Smartphone Enabled Point-of-Care Detection of Serum Markers of Liver Cancer

**Supporting Agency:** NIH, UG3CA211232

**Time Commitment:** 0.25 Academic month/1.15 Summer months (~11.6% effort)

**Performance Period:** 6/1/17-4/30/19

**Level of Funding:** $268,236 (Annual Direct Costs)

**Grants Officer:** Jo Ann S. Rinaudo, rinaudoj@mail.nih.gov, (240) 276-7133

**Goals and Specific Aims:** The overall goal of this R01 proposal is to develop a multiplexed point-of-care test (POCT) for serum markers of hepatocellular carcinoma (HCC), and to validate this POCT in the People’s Republic of China, where the incidence of HCC is disproportionately high.

**Title:** Image-guided Dosimetry for Injectable Brachytherapy based on Elastin-like Polypeptide Nanoparticles

**Supporting Agency:** NIH, 1R21CA218940

**Time Commitment:** 0.67 Academic months (~5.6% effort)

**Performance Period:** 8/1/17-7/31/19

**Level of Funding:** $130,500 (Annual Direct Costs)

**Grants Officer:** Jacek Capala, capalaj@mail.nih.gov, (240) 276-5690

**Goals and Specific Aims:** The overall objective of this proposal is to provide critical infrastructure by developing, validating, and evaluating a methodology for determining optimal image-guided dosimetry for ELP-based injectable brachytherapy.

**Title:** DMREF: Collaborative Research: High throughput Exploration of Sequence Space of Peptide Polymers that Exhibit Aqueous Demixing Phase Behavior

**Supporting Agency:** NSF, 1729671

**Time Commitment:** 0.075 Summer months (~0.6% effort)

**Performance Period:** 10/1/17-9/30/21

**Level of Funding:** $1,183,788 (Total Project Costs)

**Grants Officer:** John Schlueter, (703) 292-7766
The primary goal of the proposed research is to develop generalized materials design rules, by combining experiments, fast computer simulations and data science to accelerate the discovery and development of a potentially huge class of thermally responsive polypeptide materials. (1) Prediction of stimuli responsive behavior through machine-learning algorithms that are trained and validated by high throughout synthesis and characterization of LCST and UCST phase behavior; (2) Computational design and development of responsive 3-D polypeptide materials from building blocks from specific aim 1.

Title: A Point-of-Injury Screening Assay for Tactical Damage Control Resuscitation  
Supporting Agency: USAMRMC, DM160400  
Time Commitment: 1 Summer month (~8.3% effort)  
Performance Period: 9/15/17-9/14/20  
Level of Funding: $1,399,701 (Total Project Costs)  
Grants Officer: Thomas Winter, Sidney.t.winter.civ@mail.mil, (240) 357-1590  

Goals and Specific Aims: This proposal will develop a portable, ruggedized, handheld, and non-invasive test to be used at the point-of-injury to accomplish the following 3 tasks from a single drop of finger stick blood: blood type matching, infectious disease screening, and monitoring of lactate levels as a marker for trauma.