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Multiplex lateral flow detection and binary encoding enables a molecular colorimetric 7-segment display

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Multiplex expansion in point-of-care diagnostics usually requires a linear increase of premium commodities such as reagents or space. Here we demonstrate the power of binary and molecular encoding to compress device operations. We describe the first colorimetric 7-segment display on a paper-based biosensor, providing compact and intuitive read-outs for multiplex detections.

Multiplexing is a critical parameter for increasing diagnostic efficiency. The strategies that enable simultaneous analysis of multiple samples are largely dependent on the underlying diagnostic technology. Multiplex detection using enzyme-linked immunosorbent assays, real-time polymerase chain reactions, microarrays and/or bead-based methods (such as the Bio-Rad Bio-Plex® Systems[1] and Luminex MagPix®[2]) enable high-throughput and low volume processing, but require non-portable equipment and trained personnel for operation. For point-of-care settings, lateral flow devices (LFDs) are ideal candidates, but few commercial devices employ multiplexing. This is due to issues with specificity and reproducibility, as well as expansion limits, as described by Washburn, because the flow rate decreases with distance from the conjugate pad.[3] Here we propose a novel solution for expanding LFD multiplex detection that increases the efficiency of detection without expanding the device dimensions and consuming excess reagents. Our generic solution compresses multiplex LFD data by borrowing from computational science and applying binary encoding to create signature patterns of test dots. Thus, a sample is diagnosed based on the set of test dots that appear on the device. By judicious arrangement of test dots, we create a 7-segment LFD display that simulates digital display of information for easy interpretation by the end-user.

To develop a generic multiplex LFD, we first identified twelv individual antigen-antibody single-plex assays able to operate using a sandwich immunoassay format. In this format an analyte is sandwiched between a capture and detection antibody via tagged antigens (Fig. 1A). AuNPs conjugated to the capture antibody transforms analyte detection into a colorimetric signal. We choose fluorescein and anti-fluorescein as the common AuNP conjugated capture antibody, and identified an additional twelve commercially available antigen-antibody combinations that could potentially be used as detection antibodies (Fig. 1B). The analyte was a single-stranded DNA corresponding to a segment of the L gene of Rift Valley Fever virus.[4] Importantly, we kept the RVFV DNA analyte sequence identical in all tests (apart from the 3' antigen labels) to eliminate any differences in behavior due to subtle changes in nucleotide sequences.[5] In addition, use of a dual-labeled synthetic analyte reduced the complexity of interactions for our proof-of-concept demonstration (applied implementation would utilize labeled oligonucleotide, antibody, or aptamer binding partners to capture an unlabeled analyte). All twelve antigen-antibody pairs were effective as detection entities in the single-plex LFDs (Fig. 1 & S1), with standard LFD sensitivities as low as 5 nM.[6] We note in particular the success of labels Alexa488, Cascade Blue, Lucifer Yellow, Benzopyrene, BodipyFL, and Dansyl, which have not previously been applied in lateral flow detection.

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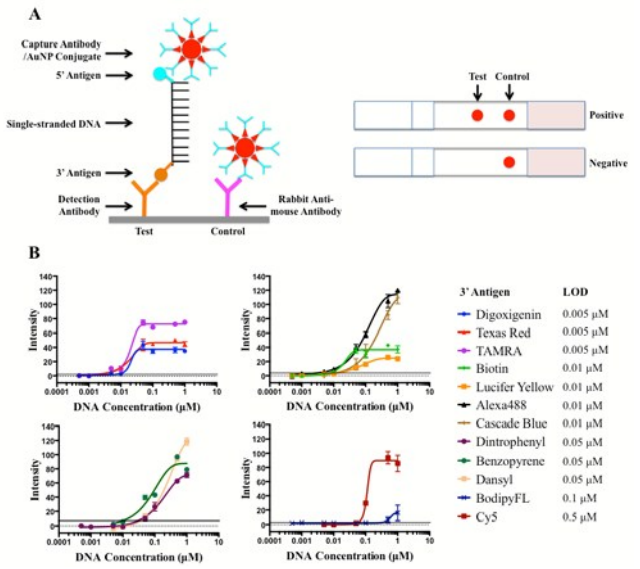

Fig. 1 (A). Design of single-plex LFDs: a dual-labeled analyte (RVFV DNA) was sandwiched between capture and detection antibodies due to antigen-antibody binding; AuNPs conjugated to the capture antibody (mouse anti-fluorescein antibody) enabled visualization of binding through the appearance of a red color at the test dot; rabbit anti-mouse antibody, which can directly bind the capture antibody (mouse anti-fluorescein), was deposited in parallel as a control. (B) Determination of detection limits for each single-plex assay: detection antibodies corresponding to the 3' antigen of dual-labeled RVFV were deposited at the test dot; resultant assay color intensity (average and standard deviation of 4 individual tests) was quantified using ImageJ software and plotted against DNA concentration; a cut-off defined as three standard deviations above the average was used to determine the limit of detection for each assay. Experimental details are described in the Supporting Information.

After demonstrating successful operation of all twelve detection antigen-antibody combinations in a single-plex LFD, we then attempted to combine these into a multiplex LFD array using a dot-matrix (3×4) format. For the arrangement of detection antibodies on the membrane, we hypothesized that the antibodies furthest from the conjugate pad would suffer the most from loss of reagents, and thus placed the least sensitive detection antibodies closest to the conjugate pad (Fig. S2A). Specificity testing indicated three non-specific reactions (Fig. S2B). To investigate the lack of binding for BodipyFL and Dansyl antigen-antibody pairs, we reduced the LFD to a 7-dot array containing six detection antibodies (chosen due to their superior specificity; see Fig. S2), with a seventh detection antibody of either anti-BodipyFL, anti-Dansyl, or anti-Cy5 detection antibody (Fig. 2A; anti-Cy5 was included as a comparative control). These were tested with a single dual-labeled analyte containing either 3' Bodipy, Dansyl, or Cy5 antigens, or with an analyte reaction mix containing the corresponding seven antigens. Interestingly, the BodipyFL antigen still did not produce any observable test dots, whereas the Dansyl test dots appeared and showed specificity in the seven antigen-antibody pairs system (Fig. 2). The lack of BodipyFL binding (Fig. 2 & S2) is most likely due to the larger surface area ablating detection, since BodibyFL was the least sensitive in the single-plex assays (Fig. 1). However, the lack of Dansyl binding in the 3x4 array but successful binding in the 7-dot array was consistent in multiple tests. This suggests the presence of other detection antibodies precluded Dansyl binding, despite it being placed in the first row of the 3x4 array. The 7-dot array that incorporated Dansyl (Fig. 2), demonstrated significant relative higher dot intensity ($p < 0.05$) compared to the Cy5 (Fig. 1 and 2), and was used for all subsequent demonstrations. These results support the notion that specificity is a hindrance in expansion into multiplexing, but with judicious design and repeated testing, compatible combinations can be identified that do not cross-react but instead reproducibly give selective responses.

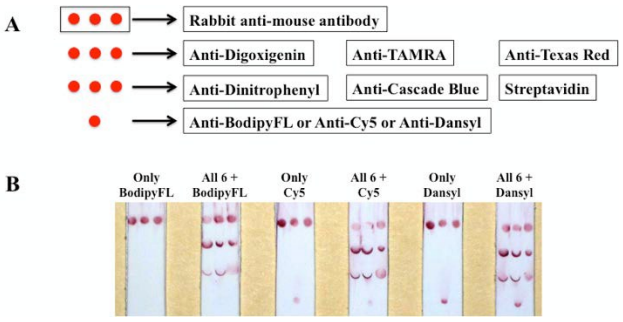

Fig. 2 Hepta-plex lateral flow detection results for combinations of seven antigen-antibody pairs. A: Positioning of detection antibodies deposited upon lateral flow strips. B: Hepta-plex lateral flow detection of 1 μM dual-labeled analyte alone (containing either 3' BodipyFL, Cy5, or Dansyl), or combinations of dual-labeled analyte (1 μM) containing 3' Biotin, Cascade Blue, Digoxigenin, Dintrophenyl, TAMRA, and Texas Red and either BodipyFL, Cy5 or Dansyl, as indicated. Assay was repeated three times and a representative photograph is shown. Experimental details are described in the Supporting Information.

On a linear scale, our 7-dot LFD array is an improvement on the current maximum multiplex detection that employs antigen-antibody reactions, since only penta-plex LFD has been previously reported.[7] However, stacking the test lines (or dots) along the flow path of the lateral flow device does not provide an intuitive result output.[8] Inspired by the electronic information display systems used in digital watches and elevators, we considered development of a proof-of-concept 7-segment LFD display. We devised a detection system to represent each segment of a 7-segment display with one detection antibody (from the seven) (Fig. 3A and S3A). Initially we used duplicate test dots to represent each segment, however, this resulted in position effects where the morphology of the second test dot was influenced by the first test dot in the vertical segments (Fig. S3). We thus reduced the vertical segments to only incorporate one

test dot (Fig. 3A). By addition of appropriate label mixtures, we consistently demonstrated clear numbers (0-9) on the 7-segment LFD display (Fig. 5B).

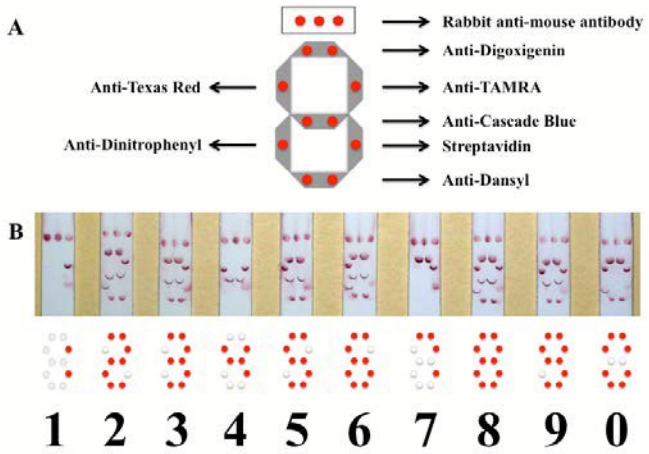

Fig. 3 7-segment display of numbers on lateral flow strips. A: Positioning of the detection antibodies to form the 7-segments of the display. B: Addition of labeled analyte signature mixtures (1 µM each) resulted in the successful appearance of numbers (0 to 9). The assay was performed three times with similar results; a photograph of one test is shown. Experimental details are described in the Supporting Information.

Our successful demonstration of the numbers 0-9 using seven antigen-antibody pairs on a LFD is the first digital-like display of numbers on a paper-based biosensor. It operates as a single-use static output that employs a pre-defined molecular encoding strategy for information transfer. The work is a significant advance on our previous molecular 7-segment display, based on molecular logic gates, which required multiple additions of samples to each segment.[5] Here the solid-phase LFD enables a single addition of sample to create a display, made possible through the novel combination of both molecular and binary encoding. This is notably different from other LFD computational strategies, which showcase embedded molecular logic gates.[9, 10] Our method also advances previous multiplex LFDs, which use multiple lines,[7, 11] bi-directional,[12, 13] parallel,[14, 15] and multi-directional systems.[16, 17] Notably, intuitive read-outs were previously proposed by Shen et al., where blood types on a paper-based biosensor were displayed using letters deposited in different sections of the biosensor (not overlayed).[18, 19] However, expansion of these multiplex LFDs is hampered by either space limitations within the original device dimension or the requirement to consume more reagents as the device size increases. In contrast, our novel combination of both binary and molecular encoding demonstrates improved multiplexing efficiency while minimizing excess reagent requirements.

The most compact LFD system to date is the microarray LFD,[20, 21] which similarly minimizes both reagent consumption and device dimension. The largest demonstration to date is detection of 384 recombinant protein antigens for analysis of human protein atlas antibody cross-reactivities.[21] Here we provide further compaction by applying binary encoding. Our generic antigen-antibody method could theoretically detect 127 (2^7-1) discrete analytes using only 7 antigen-antibody combinations. Noticeably, our system would only apply to differentiation of discrete analytes: different encoding schemes would need to be employed to differentiate analyte mixtures. However, implementation of both binary-encoded and traditional microarray LFD requires external readers to interpret results. Our 7-segment LFD intentionally restrict outputs to a numerical system to demonstrate an intuitive read-out applicable to point-of-care diagnostics that does not require external reading devices.

In this study we used an artificial ideal analyte for proof-of-concept demonstration of multiplex LFD displays. Importantly, these displays are generic and can be applied for the detection of any candidate analyte (e.g. nucleic acids, protein, lipid or small molecules) through the use of labeled binding partners. Practically, this could be implemented via labeled antibodies or aptamers for detection of their corresponding antigens. In addition, we are also considering labeled primers and probes for multiplex nucleic acid amplification, which would additionally assist with detection of low copy numbers. In this context, we note the advancement of isothermal nucleic acid amplification technologies that offer prospects for rapid point-of-care detection.[22, 23] Determination of analyte concentration could be performed using electronic reading devices (that assess intensity within a defined test area regardless of dot morphology) and by pre-training with known concentrations of analyte. Notably, implementation of pattern displays requires the use of mixtures of labeled binding partners, such that an analyte must combine with a unique subset of labels. This means that multiple individual sub-reactions must work synchronously, which ultimately increases confidence that can be drawn from a successful display.

Conclusions

Our novel 7-segment LFD enables compaction of multiplexing by borrowing from computational science and employing a binary encoding scheme that moves beyond space limitations. The easy-to-interpret results via a 7-segment display format are highly relevant to point-of-care applications. These displays are generic and can be applied for multiplex detection of any candidates (e.g. nucleic acids, protein, lipid, or small molecules) if capture reagents incorporate our specific recognition labels. This is particularly amenable for nucleic acid lateral flow, if upstream nucleic acid amplification uses primers and probes that incorporate the recognition labels.[22] Further improvements could focus on employing advanced deposition techniques to improve the performance and presentation of the outputs.[24]

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Notes and references

1. I. Bio-Rad Laboratories, Bio-Rad Bio-Plex® Systems, http://info.bio-rad.com/ww-Bio-Plex-Luminex-lp1.html?WT.srch=1&WT.mc_id=aw-pqd-AU-bioplex_luminex_gold&WT.knsh_id=631f362c-bf07-4e69-b01a-00fb1c8cb35f&gclid=COKLk_mOjcgCFYiCvQodPpEJWA, Accessed September 23rd, 2015, 2015.

2. L. Corporation, Luminex MagPix®, http://www.luminexcorp.com/clinical/instruments/magpix/, Accessed September 23rd, 2015, 2015.

3. E. W. Washburn, *Phys. Rev.*, 1921, **17**, 273-283.

4. M. Euler, Y. Wang, O. Nentwich, O. Piepenburg, F. T. Hufert and M. Weidmann, *J. Clin. Virol.*, 2012, **54**, 308-312.

5. J. Macdonald, Y. Li, M. Sutovic, H. Lederman, K. Pendri, W. Lu, B. L. Andrews, D. Stefanovic and M. N. Stojanovic, *Nano Lett.*, 2006, **6**, 2598-2603.

6. J. Li, D. McMillan and J. Macdonald, *Sensor. Mater.*, 2015, **27**, 549-561.

7. P. Noguera, G. A. Posthuma-Trumpie, M. van Tuil, F. J. van der Wal, A. de Boer, A. P. H. A. Moers and A. van Amerongen, *Anal Bioanal Chem*, 2011, **399**, 831-838.

8. J. E. Poje, T. Kastratovic, A. R. Macdonald, A. C. Guillermo, S. E. Troetti, O. J. Jabado, M. L. Fanning, D. Stefanovic and J. Macdonald, *Angew. Chem. Int. Ed. Engl.*, 2014, **53**, 9222-9225.

9. J. Chen, Z. Fang, P. Lie and L. Zeng, *Anal. Chem.*, 2012, **84**, 6321-6325.

10. Y. Huang, W. Wen, D. Du, X. Zhang, S. Wang and Y. Lin, *Biosens. Bioelectron.*, 2014, **61**, 598-604.

11. L. Tian, T. Sato, K. Niwa, M. Kawase, A. C. Tanner and N. Takahashi, *Biomed. Res. Int.*, 2014, **2014**, 180323.

12. S. M. Hossain, C. Ozimok, C. Sicard, S. D. Aguirre, M. M. Ali, Y. Li and J. D. Brennan, *Anal Bioanal Chem*, 2012, **403**, 1567-1576.

13. S. M. Hossain, R. E. Luckham, M. J. McFadden and J. D. Brennan, *Anal. Chem.*, 2009, **81**, 9055-9064.

14. D. a. C. Becton, BD™ EZ Flu A+B Test, http://www.bd.com/ds/productCenter/256050.asp, Accessed January 12th, 2015.

15. A. Technologies, RAID, http://www.alexeter.com/biow/Products/products/strips/RAID_DX.asp, Accessed January 12th, 2015.

16. C. Z. Li, K. Vandenberg, S. Prabhulkar, X. Zhu, L. Schneper, K. Methee, C. J. Rosser and E. Almeide, *Biosens. Bioelectron.*, 2011, **26**, 4342-4348.

17. K. L. Peters, I. Corbin, L. M. Kaufman, K. Zreibe, L. Blanes and B. R. McCord, *Anal. Methods.*, 2015, **7**, 63-70.

18. M. Li, J. Tian, M. Al-Tamimi and W. Shen, *Angew. Chem. Int. Ed. Engl.*, 2012, **51**, 5497-5501.

19. M. Li, W. L. Then, L. Li and W. Shen, *Anal Bioanal Chem*, 2014, **406**, 669-677.

20. J. Gantelius, C. Hamsten, M. Neiman, J. M. Schwenk, A. Persson and H. Andersson-Svahn, *J. Microbiol. Methods.*, 2010, **82**, 11-18.

21. J. Gantelius, T. Bass, R. Sjoberg, P. Nilsson and H. Andersson-Svahn, *Int. J. Mol. Sci.*, 2011, **12**, 7748-7759.

22. J. Li and J. Macdonald, *Biosens. Bioelectron.*, 2014, **64C**, 196-211.

23. A. James and J. Macdonald, *Expert. Rev. Mol. Diagn.*, 2015, **15**, 1475-1489.

24. J. Li, F. Rossignol and J. Macdonald, *Lab Chip.*, 2015, **15**, 2538-2558.