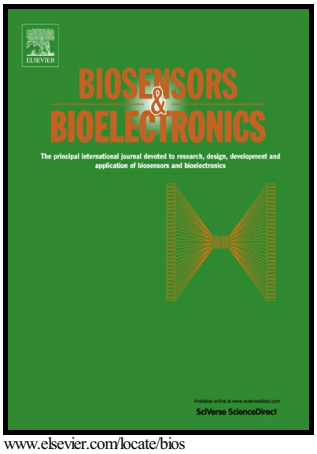

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Multiplexed lateral flow biosensors: technological advances for radically improving point-of-care diagnoses

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Abstract

Lateral flow biosensors are a leading technology in point-of-care diagnostics due to their simplicity, rapidness and low cost. Their primacy in this arena continues through technological breakthroughs such as multiplexing: the detection of more than one biomarker in a single assay. Multiplexing capacity is critical for improving diagnostic efficiency, enhancing the diagnostic precision for specific diseases and reducing diagnostic cost. Here we review, for the first time, the various types and strategies employed for creating multiplexed lateral flow biosensors. These are classified into four main categories in terms of specific application or multiplexing level, namely linear, parameter, spatial and conceptual. We describe the practical applications and implications for each approach and compare their advantages and disadvantages. Importantly, multiplexing is still subject to limitations of the traditional lateral flow device, such as sensitivity and specificity. However, by pushing the limitations of the traditional medium into the multiplex arena, several technological breakthroughs are emerging with novel solutions that further expand the utility of lateral flow biosensing for point-of-care applications.

Contents

1. Introduction

Multiplex testing is becoming indispensible in contemporary clinical diagnoses. With the increasing numbers of (bio)markers discovered, there is often a need to detect several (bio)markers simultaneously to generate meaningful or conclusive information. This is important for reliable disease detection and monitoring (e.g.

cancer), as a single (bio)marker may be indicative of more than one disease, with a statistically inadequate predictive value (Rusling et al., 2010). In addition, false positives and negatives may appear quite frequently with single (bio)marker detection, but could be minimised by detecting a (bio)marker panel (Rusling et al., 2010). Therefore multiplex testing ensures precise diagnostics and mitigates patient risk. Another benefit of multiplex testing is the reduction of diagnostic time and costs in comparison to performing multiple single tests.

Many multiplex testing methods have been developed for diagnostics. The commonly used ones include multiplex real-time polymerase chain reaction (PCR) (Elnifro et al., 2000), enzyme-linked immunosorbent assay (ELISA) (Tighe et al., 2015), microarrays (Moore et al., 2016; Yoo et al., 2009) and next-generation sequencing (Xuan et al., 2013). Although these multiplex testing methods are sensitive and specific, and increase diagnostic efficiency, they require (1) sophisticated and expensive equipment, which are not often available in low resource-setting areas; (2) multiple and lengthy (e.g. several hours) operation procedures; and (3) skilled technicians to perform and analyse the test. These requirements make these multiplex testing methods not favorable for point-of-care (POC) diagnostics that fit with the ASSURED criteria (Affordable, Sensitive, Specific, User-friendly, Rapid/Robust, Equipment-free and Deliverable to end users). In comparison to these methods, multiplexed lateral flow biosensors are promising POC candidates that fulfill the ASSURED criteria perfectly (Li and Macdonald, 2016).

Multiplexed lateral flow biosensors are the multiple analyte version of the traditional single-plex lateral flow biosensor. Lateral flow biosensors are self-operating devices that perform rapid assays on a membrane in a chromographic manner from a single sample addition. The sample encounters a series of reaction zones as it migrates across the membrane, which results in a readable output in these reaction zones. Lateral flow biosensors are simple, rapid (test done within minutes), low cost and can be used in the vicinity of the customer. The most famous example of a lateral flow biosensor is the at-home pregnancy test, which was also the first commercialised product of the lateral flow biosensor launched in 1988 (Chard, 1992). Since then, applications for lateral flow devices have included the detection of parasites (Mens et al., 2012a), bacteria (Blažková et al., 2009a; Chua et al., 2011; Pohlmann et al., 2014;

Roskos et al., 2013), cells (Liu et al., 2009; Wu et al., 2013), viruses (Chowdry et al., 2014; Deng et al., 2014; Peng et al., 2008; Xiang et al., 2012), toxins in food (Liu et al., 2008), environmental samples (Blažková et al., 2009c; Guo et al., 2009; Zhou et al., 2004), illicit drugs (Li et al., 2007), hormones (Leung et al., 2003) and biological markers (Li et al., 2010; Xu et al., 2009).

Multiplexed lateral flow biosensors are emerging in response to the abovementioned corresponding clinical and industrial needs. However, a critical question is how many different analytes can be detected (simultaneously) within a single lateral flow biosensor at once? This question needs to be critiqued in the context of two limiting factors. The first limitation is that simply enlarging a device may not be economical, as a larger device will consume more reagents that can actually increase costs. The second limitation is a consideration of Washburn's theory, which describes that the capillary flow rate is proportional to the square root of time (Washburn, 1921): the further the test line is from the origin, the exponentially longer it takes a sample to wick to the test line. Therefore, multiplexing capability must be considered within the trade-off between space (device dimension), assay time and the number of analytes detected.

Here we provide the first review of multiplexed lateral flow biosensors, which begins with the typical configuration and operation of the traditional single-plex lateral flow biosensor before describing multiplexed lateral flow biosensors. This review is intended to provide readers with a conceptual outline of the different types of multiplexed lateral flow biosensors published in the literature to date, and describe and compare their performance, including sensitivity, specificity and quantitative analysis. We classify these published multiplexed lateral flow biosensors based on four categories in terms of specific application or multiplexing level: (1) multiple analyte detection on a single device; (2) multi-parameter detection; (3) spatial multiplexity – 2 dimensional paper network (2DPN); and (4) conceptual multiplexing. In addition, we assess future perspectives for multiplex lateral flow biosensor development and applications within the limitations of the overall technology, particularly in regards to factors that affect sensitivity and specificity. These factors in particular are critical for further development of lateral flow biosensors and expanding their reach within POC diagnostics.

2. Configuration and operation of lateral flow biosensors

The typical configuration of a lateral flow biosensor consists of a membrane and several functional pads that are connected to each other and assembled on an adhesive backing card (Fig.1A). The sample pad is for loading sample and ensures even distribution of sample solution to downstream components. The conjugate pad is adhered next to the sample pad, and is often the place where analyte detection molecule/tracer conjugates are deposited. Its main function is to control the releasing of reactants solution onto the membrane and hold the reactants stable over their entire shelf-life (Brendan, 2009). Gold nanoparticles are the most commonly used tracer, but tracers can also be made from latex beads (Mao et al., 2013b), carbon nanomaterials (nanoparticles (Posthuma-Trumpie et al., 2012), nanostrings (Kalogianni et al., 2011) and nanotubes (Qiu et al., 2015)), silica nanoparticles (Bamrungsap et al., 2013), polystyrene microspheres (Kalogianni et al., 2009), quantum dots (Zou et al., 2010) and up-converting phosphor (Niedbala et al., 2001). The membrane (e.g. nitrocellulose membrane) is pre-deposited with other detection molecules (e.g. antibody or oligonucleotide) and adhered next to the conjugate pad; this section is where analyte detection takes place. Finally, the absorbent pad is used to enhance the capillary driving force and absorb all the unreacted substances.

(Insert Figure 1 Here)

Operation of a typical single-plex lateral flow biosensor starts with loading the analyte (in a solution) to the sample pad (Fig. 1B(i)); the analyte will move along the biosensor via capillary force. Once the analyte reaches the conjugate pad, it is captured by the capture antibody/tracer conjugates (e.g. Anti-analyte antibody 1/tracer; Fig. 1B(ii)), and the resulting complexes keep moving along until they are detected at the test line via a pre-deposited detection antibody (e.g. Anti-analyte Antibody 2; Fig. 1B(iii)). The unreacted antibody/tracer conjugates will finally be detected at the control line by a species-specific antibody (e.g. Antibody 3; Fig. 1B(iii); any unreacted reactants are absorbed by the absorbent pad. Note that the capture and detection antibodies bind to the analyte (e.g. antigen) at different epitopes (for a typical lateral flow sandwich assay). Observation of both a coloured test line

and a coloured control line on the membrane is a positive result (Fig. 1B(iv)). A single coloured control line is a negative result (Fig. 1B(iv)). Appearance of no coloured lines at all is an invalid result (Fig. 1B(iv)) and the test must be repeated. The control line indicates that the sample has migrated across the membrane as intended, regardless of whether the analyte is present in the sample.

3. Multiplexed lateral flow biosensors

Multiplexed lateral flow biosensors allow simultaneous detection of multiple analytes on a single device. Increasing lateral flow multiplexing capacity is an exquisite art of accommodating as many test lines (or dots) as possible, within a limited space on a device. In addition, the types of analytes detected are also a key consideration during development. In this section, we describe different multiplexing strategies and give comprehensive descriptions and comparisons of these published strategies. A summary of the different multiplexing conceptualisations and their performance is provided in Table 1, and an overview of the different technologies discussed is provided in Figure 1C.

3.1. Multiple analytes detection on a single device

Traditional lateral flow biosensors provide single-plex detection comprised of a test plus a control lines or dots as the result output. The appearance of the test line/dot indicates the detection of a specific analyte, and the control line/dot serves to validate the test. However, the number of biomarkers linked to certain diseases is often increasing, with multiple biomarkers being assayed to confirm diagnosis. For example, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2 or neu), tumor protein p53 (p53) and Ki-67 are traditional biomarkers for prognostic assessment of breast cancer (Malinowski, 2007); The combinatorial detection of UDP-N-Acetyl-alpha-D-galactosamine transferase (GalNAc-T3), prostate-specific membrane antigen (PSMA), Hepsin and prostate cancer antigen 3 (PCA3 or DD3) showed improved sensitivity for prostate cancer diagnosis in comparison to the detection of the single conventional prostate specific antigen (PSA) biomarker (Landers et al., 2005). This example demonstrates how single-plex test kits are less efficient for diagnoses. Accordingly, the most direct way

to improve the efficiency of lateral flow diagnostics is to facilitate the simultaneous detection of multiple analytes on a single device. The strategies employed to enable such multiplexing within a lateral flow biosensor are diverse, ranging from mono- or multi- coloured lines or dots, parallel or non-parallel by device arrangement, as well as mono-, bi- or multi- directional by device structure. We have considered all the multiplex lateral flow biosensors for multi-analyte detection and considered their mode of operation, as outlined in Figure 2, and detailed from Section 3.1.1. to Section 3.1.6..

(Insert Figure 2 Here)

3.1.1. Single lateral flow biosensor with multiple mono-coloured lines or dots in a single array

Combining multiple lines or dots in an array is the most direct way of increasing the detection capacity while retaining the merits of single lateral flow detection systems (Fig. 2A). This approach has been mostly used in the detection of food borne bacterial pathogens (Blažková et al., 2009a; Blažková et al., 2009b; Noguera et al., 2011; Tian et al., 2014) and mycotoxins (Huang et al., 2012; Kolosova et al., 2007; Lattanzio et al., 2012; Shim et al., 2009) but has also been described for the detection of single nucleotide polymorphisms (SNPs) (Konstantou et al., 2009; Litos et al., 2009), parasites (Mens et al., 2012b), papilloma virus (Xu et al., 2014) and antibodies (anti-rabbit IgG and anti-human IgM) (Mao et al., 2013a). The maximum multiplex detection capacity (number of test line or dot) reported for this form of lateral flow biosensor version using colourimetric detection is five (penta-plex) (Noguera et al., 2011; Tian et al., 2014). Whereas, using fluorescent detection, the maximum multiplex detection ability is thirteen (trideca-plex) (Xu et al., 2014). In their biosensor system, thirteen different types of papilloma virus were detected using only two fluorescent labels (6-carboxyfluorescein – FAM and 6-Carboxyl-X-Rhodamine – ROX; the FAM and the ROX were applied in a staged manner in the test zones) (Xu et al., 2014).

Commercially, the maximum detection capacity available using nucleic acids as analytes is the duplex detection of Milenia HybriDetect 2T kit (Biotec, 2016). For the

detection of non-nucleic acids, the maximum detection capacity is the triplex detection of SD BIOLINE Influenza Ag A/B/A(H1N1) virus A pandemic kit in which the analytes are the antigens haemagglutinin or envelope proteins (Alere, 2015).

Expanding multiplex detection by adding more lines or dots in a single array along the device is extremely limited. This is because the capillary flow time is proportional to the exponential of the distance, and as more analytes are included, the assay time will increase to reach the furthest test line.

3.1.2. Single lateral flow biosensor with multi-coloured lines or dots

An extension of the multiple test process to distinguish between analytes involves the use of different coloured tracers (Fig. 2B). In particular, nanomaterials can be synthesised/designed to produce different colours and/or morphologies by adjusting the reaction conditions (e.g. pH, temperature and reagent concentration) (Link and El-Sayed, 1999; Premkumar et al., 2011; Sun and Xia, 2002; Zhou et al., 2008). The idea of multiplex detection with different coloured dots was first demonstrated by Panfilova et al. (Khlebtsov and Khlebtsov, 2012; Panfilova et al., 2012), who synthesised silver nanocubes (yellow), gold/silver alloy nanoparticles (red) and gold/silver nanocages (blue), and used these nanomaterials to label chicken IgG, rat IgG and mouse IgG, respectively for the detection of their corresponding antibodies. The multiplex detection with multi-colour dots was successful and showed no interference between the colourimetric signals. Yen et al. (Yen et al., 2015) also synthesised different coloured silver nanoparticles for the successful detection of dengue (green silver nanoparticles), yellow fever (orange silver nanoparticles) and Ebola (Zaire strain; red silver nanoparticles). In addition to confirming the observations of Panfilova et al., Yen et al. (2015) demonstrated the detection of the three viruses in one test line. The resulting colour was brown due to the combination of the green, orange and red silver nanoparticles. In addition to the silver nanomaterials, the fluorescent nanocrystals - quantum dots were successful in the detection of three antibiotics (ofloxacin (red), chloramphenicol (yellow) and streptomycin (green)) in milk, the so-called “traffic light assay” (Taranova et al., 2015).

3.1.3. Single lateral flow biosensor with multiple dots as arrays

Beyond displaying a single set of lines or dots, another multiplexing alternative is to display a series of lines or dots in a format akin to a small microarray. In this way, the multiplexing capacity can simultaneously detect several dozens of analytes (Fig. 2C). This has been demonstrated for the detection of multiple mutations (Elenis et al., 2011), illicit drugs (Taranova et al., 2013), antibodies (IgE and IgG) (Chinnasamy et al., 2014; Gantelius et al., 2011), recombinant protein antigens (Gantelius et al., 2010) and protein biomarker (Cartilage oligomeric matrix protein) (Hong et al., 2012). The highest multiplexing ability is the simultaneous detection of 384 dots on a single lateral flow dipstick demonstrated by Gantelius and co-workers (Gantelius et al., 2011).

The multiplexing ability of the lateral flow microarray version can be further expanded with the help of delicate reagent dispensing technology, such as inkjet printing. Inkjet printing dispenses reagents (including biomolecules) in nano- or pico-liter volumes with high precision, resolution and robustness (Cooley et al., 2001; Delaney et al., 2009; Li et al., 2015; Sirringhaus and Shimoda, 2003; Xu et al., 2005). However, a potential problem for the lateral flow microarray is that as the density of the test dots increases (that eye-only observing cannot interpret), more sophisticated equipment is needed to read and interpret the results. Such sophisticated readers are not yet available, however, SCIENION and Axxin companies announced a collaboration in September 2015 to develop a reader system for multiplexed lateral flow microarrays (SCIENION, 2015).

3.1.4. Multiple lateral flow biosensors with single line – parallelism

An alternative form of multiplexing is parallelism, which involves combining multiple single lateral flow biosensors within one plastic cassette. While these devices have not been discussed and evaluated in the scientific literature, they remain one of the few multiplexing strategies to be adopted commercially (apart from the Milenia Hybridtect kit described in Section 3.1.1.) (Fig. 2D). Available kits include the detection of influenza (e.g. BD™ Directigen™ EZ Flu A+B kit (Becton, 2014) and Alere BinaxNOW® Influenza A&B Card (Alere, 2010-2015), as well as bacteria

toxins (RAID 8 and RAID TOX (Technologies, 2014)). Such multiplex lateral flow detection developments may alleviate cross-reaction problems (e.g. the interference between different analytes in a mixture). However, there is an increase in the device size dimension and reagents consumption as multiplex detectability expands.

3.1.5. Bi-directional lateral flow biosensor

As an interesting development of the basic multiplex lateral flow method (Section 2.1 version), the bi-directional lateral flow dipstick employs a second lateral flow by inverting the dipstick once the first round running reaches a certain threshold (Fig. 2E). Brennan and his co-workers (Hossain et al., 2009; Hossain et al., 2012) indicate their bi-directional system is advantageous because the lateral flow dipstick: (1) alleviates cross-reaction problems as independent analyte detections take place at different ends of the dipstick (Hossain et al., 2012); and (2) the analyte or certain reagents can be pre-mixed with each other for a certain period of time to provide an essential reaction condition for the final signal production (Hossain et al., 2009).

3.1.6. Multi-directional lateral flow biosensor with single line or dot

Another multi-directional method uses additional "arms" or reaction zones to increase the detection capacity, such as the multi-directional lateral flow dipstick (Fig. 2F). The additional reaction zones provide: (1) more test lines or dots; (2) the simultaneous and independent detection on each arm or zone; and (3) a self-calibration system, where information on multiple interactions (such as in a serial dilution pre-deposited within different arms) can be collected at the same time. This allows the generation of a standard curve for quantitative analysis, which minimises environmental condition effects and avoids systematic errors (Wang et al., 2010; Zhu et al., 2014). In comparison to parallelism (Fig. 2D), the multi-directional lateral flow dipstick integrates multiple detections from a single sample inlet. However, as with parallelism, increases reagents usage.

Table 1 Summary of important parameters of multiplexed lateral flow biosensors

Multiplex Category	Higest Multiplexing (by detection of different analytes)	Analyte(s)	Tracer(s)	Detection Mode	Detection Method	Analytical Sensitivities	Application	References
Multiple analytes detection on a single device with multiple mono-coloured lines	Trideca-plex (13)	Human papillomavirus (HPV) nucleic acids	Fluorescent Labels: FAM and ROX	Oligonucleotide hybridisations	Fluorescent	10 - 100 copies plasmid DNA/μL	Virus detection	(Xu et al., 2014)
Multiple analytes detection on a single device with multiple mono-coloured lines	Tetra-plex (4)	Genotyping of two biallelic polymorphisms of MBL2 gene	Gold nanoparticles	Oligonucleotide hybridisations	Colourimetric	NA	Single-nucleotide polymorphisms (SNP) detection	(Litos et al., 2009)
Multiple analytes detection on a single device with multiple mono-coloured lines	Penta-plex (5)	Plaque bacteria: Streptococcus mutans, Streptococcus sobrinus, Scardovia wiggsiae, Actinomyces species and Veillonella parvula nucleic acids	Latex bead	Oligonucleotide hybridisations	Colourimetric	100 pg for all the analytes	Bacteria detection	(Tian et al., 2014)
Multiple analytes detection on a single device with multiple mono-coloured lines	Penta-plex (5)	Shiga toxin-producing Escherichia coli (E. coli) virulence factors (vt1, vt2, eae and ehxA) and 16S control (specific for E. coli) nucleic acids	Carbon nanoparticles	Antigen-antibody interactions	Colourimetric	vt1 (4.1 x 10^5 cfu/mL), vt2 (6.7 x 10^4 cfu/mL), eae (3.7 x 10^5 cfu/mL), ehxA (1.4 x 10^5 cfu/mL) and 16S (1.8 x 10^5 cfu/mL)	Bacterial toxin detection	(Noguera et al., 2011)
Multiple analytes detection on a single device with multiple mono-coloured lines	Triplex (3)	Foot-and-mouth disease virus serotype O, A and Asia 1 antigens	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	Serotype O (viral particles/test), serotype A (3400 viral particles/test) and Asia 1 (7200 viral particles/test)	Virus detection	(Yang et al., 2015)
Multiple analytes detection on a single device with multiple mono-coloured lines	Tetra-plex (4)	Hepatitis B virus genotyping A, B, C and D surface antigens	FluoroMax fluorescent nanoparticles	Antigen-antibody interactions	Fluorescent	Genotype A (2.5 IU/mL), genotype B (5 IU/mL), genotype C (5 IU/mL) and genotype D (10 IU/mL)	Virus detection	(Song et al., 2015)
Multiple analytes detection on a single device with multiple mono-coloured lines	Triplex (3)	Acquired immune deficiency syndrome (AIDS) (anti-HIV IgG), hepatitis C virus (HCV) (anti-HCV IgG) and hepatitis A virus (HAV) (anti-HAV IgG and IgM)	Proteinticles	Antigen-antibody interactions	Colourimetric	NA	Virus detection	(Lee et al., 2015b)

Table 1 (continuous) Summary of important parameters of multiplexed lateral flow biosensors

Multiplex Category	Higest Multiplexing (by detection of different analytes)	Analyte(s)	Tracer(s)	Detection Mode	Detection Method	Analytical Sensitivities	Application	References
Multiple analytes detection on a single device with multiple multi-coloured lines	Triplex (3)	Dengue virus (DENV) NS1 protein, Yellow Fever virus (YEV) NS1 protein and Ebola virus, Zaire strain (ZEBOV) glycoprotein	Silver nanoparticles	Antigen-antibody interactions	Colourimetric	All in the range of 150 ng/mL	Virus detection	(Yen et al., 2015)
Multiple analytes detection on a single device with multiple multi-coloured lines	Triplex (3)	Ofloxacin, chloramphenicol and streptomycin	Quantum dots	Antigen-antibody interactions	Colourimetric	Ofloxacin (0.3 ng/mL), chloramphenicol (0.12 ng/mL) and streptomycin (0.2 ng/mL)	Antibiotics detection	(Taranova et al., 2015)
Multiple analytes detection on a single device with multiple multi-coloured lines	Triplex (3)	Rabbit anti-chicken, anti-rat and anti-mouse antibodies	Silver nanocubes, gold/silver alloy nanoparticles and gold/silver nanocages	Antigen-antibody interactions	Colourimetric	NA	Antibody detection	(Panfilova et al., 2012)
Multiple analytes detection on a single device with multiple dots as arrays	Pentadeca- (15)	15 specific human IgE responses (with 4 downstream identical spots for each allergen and 3 rows of human IgE as a positive control)	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	NA	Antibody detection	(Chinnasamy et al., 2014)
Multiple analytes detection on a single device with multiple dots as arrays	Tetra-plex (4)	Morphine, benzoylecgonine, amphetamine and methamphetamine (8 duplicates of each drugs)	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	Morphine (2.0 ng/mL), benzoylecgonine (3.2 ng/mL), amphetamine (1.2 ng/mL) and methamphetamine (20.1 ng/mL)	Drug of abuse detection	(Taranova et al., 2013)
Multiple analytes detection on a single device with multiple dots as arrays	Deca-plex (10)	TLR4 gene fragment (containing the A896G and C1196T SNPs with 5 normal types and 5 mutant types)	Gold nanoparticles	Oligonucleotide hybridisations	Colourimetric	NA	Multiallele detection	(Elenis et al., 2011)
Multiple analytes detection on a single device with multiple dots as arrays	26-plex	26 individual HPA antibodies and 8 concurrent specific IgG (on a 24 x 16 microarrary)	Gold nanoparticles	Oligonucleotide hybridisations	Colourimetric	NA	Antibody detection	(Gantelius et al., 2011)

Table 1 (continuous) Summary of important parameters of multiplexed lateral flow biosensors

Multiplex Category	Higest Multiplexing (by detection of different analytes)	Analyte(s)	Tracer(s)	Detection Mode	Detection Method	Analytical Sensitivities	Application	References
Multiple lateral flow biosensors with single line – parallelism	Duplex (2)	Influenza A and B viral antigens	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	NA	Virus detection	(Becton, 2014)
Multiple lateral flow biosensors with single line – parallelism	Octa-plex (8)	Bacteria Toxins (Bacillus anthracis, Ricin, Botulinum toxin, Staphylococcal enterotoxin B, Plague, Brucella, Tularemia and Orthopox)	NA	Antigen-antibody interactions	Colourimetric	NA	Bacterial toxin detection	(Technologies, 2014)
Bi-directional lateral flow biosensor	Duplex (2)	E. coli BL21 (non-pathogenic) and E. coli O157:H7 (pathogenic)	Enzyme and substrates	Enzymatic reaction	Colourimetric	E. coli BL21 (~20 colony-forming units (cfu)/mL) and E. coli O157:H7 (5 cfu/mL)	Bacteria detection	(Hossain et al., 2012)
Bi-directional lateral flow biosensor	Tetra-plex (4)	Organophosphate pesticides: bendiocarb, carbaryl, paraoxon and malathion	Enzyme and substrates	Enzymatic reaction	Colourimetric	Bendiocarb (~1 nM), carbaryl (~10 nM), paraoxon (~1 nM) and malathion (~10 nM)	Pesticides detection	(Hossain et al., 2009)
Multi-directional lateral flow biosensor with single line or dot	Hepta-plex (7)	Heavy metals: Hg(II), Ag(I), Cu(II), Cd(II), Pb(II), Cr(VI), Ni(II)	Enzyme and substrates	Enzymatic reaction	Colourimetric	Hg(II) (0.001 ppm), Ag(I) (0.002 ppm), Cu(II) (0.020 ppm), Cd(II) (0.020 ppm), Pb(II) (0.140 ppm), Cr(VI) (0.150 ppm), Ni(II) (0.230 ppm)	Heavy metals detection	(Hossain and Brennan, 2011)
Multi-directional lateral flow biosensor with single line or dot	Penta-plex (5)	Inorganic explosives: chlorate, nitrate, nitrite, ammonium and perchlorate	Enzyme and substrates	Enzymatic reaction	Colourimetric	Inorganic explosives: chlorate (2.64 μg), nitrate (21.12 μg), nitrite (2.64 μg), ammonium (7.92 μg) and perchlorate (10.56 μg)	Inorganic explosives detection	(Peters et al., 2015)
Multi-directional lateral flow biosensor with single line or dot	Duplex (2)	Pseudomonas aeruginosa and staphylococcus aureus	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	500-5000 cfu/mL	Bacteria detection	(Li et al., 2011)

Table 1 (continuous) Summary of important parameters of multiplexed lateral flow biosensors

Multiplex Category	Higest Multiplexing (by detection of different analytes)	Analyte(s)	Tracer(s)	Detection Mode	Detection Method	Analytical Sensitivities	Application	References
Multi-directional lateral flow biosensor with single line or dot	Single-plex (1)	Glucose (dilutions from 11.0 x 10^{-3} to 1.0 x 10^{-3} M)	Enzyme and substrates	Enzymatic reaction	Colourimetric	3 x 10^{-4} M	Gluccose detection	(Zhu et al., 2014)
Multi-parameter lateral flow detection with self-calibration	Duplex (2)	Treponema pallidum (TP) antibody and Type B hepatitis surface antigen	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	TP (8-fold diluted standard) and Type B hepatitis surface antigen (5 ng/mL)	Bacterium and virus detections	(Oku et al., 2001)
Multi-parameter lateral flow detection	Duplex (2)	60-mer DNA and rabbit IgG	Gold nanoparticles	Antigen-antibody interaction and oligonucleotide hybridisation	Colourimetric	60-mer DNA (0.5 nM) and rabbit IgG (2 ng/mL)	Nucleic acid and antibody detections	(Mao et al., 2014)
2D Paper network	Single-plex (1)	Malaria protein: plasmodium falciparum histidine-rich protein 2 (pfHRP2)	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	pfHRP2 (3.6 ng/mL; in comparison to unenhanced signal result, 10 ng/mL)	Parasite detection	(Fridley et al., 2014)
2D Paper network card	Single-plex (1)	Malaria protein: pfHRP2	Gold nanoparticles	Antigen-antibody interactions	Colourimetric	pfHRP2 (2.9 ± 1.2 ng/mL)	Parasite detection	(Fu et al., 2012)
Logic gate-based lateral flow detection	Single-plex (1)	Thrombin and adenosine triphosphate (ATP)	Gold nanoparticles	Aptamer and small molecule interactions	Colourimetric	NA	Protein and small molecule detections	(Chen et al., 2012)
Logic gate-based lateral flow detection	Single-plex (1)	Carcinoembryonic antigen (CEA) protein and c-DNA	Gold nanoparticles	Oligonucleotide hairpin and protein interatction and oligonucleotide hybridisations	Colourimetric	NA	Protein and nucleic acid detections	(Huang et al., 2014)

NA = not available

3.2. Multi-parameter lateral flow detection

The multiplex lateral flow detections described in Section 3.1. have all been applied to the detection of a single type of analyte (e.g. either nucleic acids or proteins). However, the simultaneous detection of different types of analytes (e.g. nucleic acid and proteins on a single device) has also been performed. This is highly relevant for personalised diagnostics, as the detection of multiple types of disease biomarkers is critical in certain diseases or multi-syndromic patients. The multi-parameter lateral flow biosensor was originally prototyped by Oku et al. (Oku et al., 2001). Their device was designed to simultaneously detect Treponema pallidum (TP) antibody and Type B hepatitis (HB) surface antigen. The TP antibody and HB antigen were captured by TP antigen/tracer or anti-HB antibody/tracer conjugates, and were subsequently detected by TP antigen/oligonucleotide or anti-HB antibody/oligonucleotide conjugates in the test zones (Fig. 3A). The two analytes detection depended on antigen-antibody interactions and thus cross-reaction/interference is a potential problem. However, Oku et al. (2001) found their detection system was not subject to this interference. Detection results from their multi-parameter lateral flow biosensor were identical to those of the commercially available lateral flow kit. The development of the multi-parameter lateral flow biosensor had not been followed up until recently when Mao et al. (Mao et al., 2014) developed a lateral flow biosensor for the simultaneous detection of a nucleic acid (60-mer DNA) and a protein (rabbit IgG). Their detection system differed from that of Oku et al. by adopting both the antigen-antibody interaction and oligonucleotides hybridisations (Fig. 3B). Thus, in their research, the major consideration was optimising the different kinetics between the two detection systems. Mao et al. found that optimising the amount of the detection reagents (oligonucleotide probe and antibody) and the running buffer components were helpful to solve this problem (Mao et al., 2014).

(Insert Figure 3 Here)

Oku et al.'s and Mao et al.'s work are the only two papers that describe the development of multi-parameter lateral flow biosensor, and even then, the number of different types of analytes detected was only two. While developing the multi-

parameter lateral flow detection is challenging, it is highly relevant for improving the precision of diagnosing a disease or detecting a pathogen, as a single parameter (biomarker) detection may not provide adequate accuracy. Indeed, the multi-parameter detection is very important for the diagnosis and treatment of genetic diseases, where the nucleic acid-nucleic acid, protein-protein and/or nucleic acid-protein interactions provide associated key diagnostic information (Anker et al., 2003; Flamini et al., 2006; Swarup and Rajeswari, 2007).

3.3. Spatial multiplexity – 2D paper network

Lateral flow biosensors can also be multiplexed by extending to a two-dimensional format. This was developed by Paul Yager and his co-workers, and they named their novel lateral flow biosensor as a two-dimensional paper network – 2DPN (Fu et al., 2010a; Fu et al., 2010b). The 2DPN connects multiple inlets to a single detection zone in comparison to the conventional lateral flow biosensor that connects multiple detection arms or zones to a single inlet. The 2DPN strategy is particular useful for the signal amplification process on lateral flow biosensors (i.e., for improving analytical sensitivity). Indeed, signal amplification is an important component to improving analytical sensitivity of the lateral flow biosensor. Many methods for signal amplification of lateral flow biosensors have been published in the literature, such as using thermal contrast (Qin et al., 2012), by pre-concentrating the analyte (Mashayekhi et al., 2010) and using a "bridging antibody" to load more signalling molecules (Rivas et al., 2015). However, the most commonly used amplification strategies are enzymes (Parolo et al., 2013) and silver (Yang et al., 2011). In these cases, additional running steps are required to deliver specific solutions (e.g. substrates) that are essential to amplify the signal to the test line(s), which increases the complexity of performing lateral flow detection. The 2DPN by Yager et al. eliminates these multi-step processing (washing and adding signal amplification reagents) and combines them into a single lateral flow running step. The reagents required for washing and signal enhancing can be sequentially delivered to the detection zone in an automated manner with only a single activation step. Although this prototype was successful, it still required a multi-channel dispenser to simultaneously dispense the reagents to the three inlets to initiate the detection (Fig. 4A).

To address this problem, Yager and his co-workers modified the existing 2DPN to a 2DPN card. In the 2DPN card system, the conjugate pad(s) or the sample pad(s) were no longer connected to the multiple inlets, instead these pads were placed separately in defined regions within a plastic cassette (Fig. 4B). The detection was initiated by dispensing required reagents to these pads followed by folding the plastic cassette to allow the reagent wick to the detection zone through the main inlet (Fu et al., 2011; Fu et al., 2012). In this case, the reagent application starting time was controllable and a multi-channel dispenser was unnecessary.

(Insert Figure 4 Here)

While the 2DPN card system showed a controlled reagent application starting time, the system was complicated due to the pad placement. On account of the porosity of the nitrocellulose membrane and the inkjet printing technology, Yager and his co-worker directly inkjet patterned all the reagents to their desired regions on a nitrocellulose membrane. The new system enabled controlled rehydrating of the printed reagents during capillary flow (Fig. 5). The inkjet patterned reagents also alleviated the requirement to freshly pre-mix the three components of the signal enhancing solution step as they were inkjet printed next to each other on the nitrocellulose membrane, and could be automatically mixed to each other as the buffer gradually wicked up (Fig. 5) (Fridley et al., 2014). In addition, this approach proved to be a good prototype towards developing roll-to-roll fabrication of the 2DPN lateral flow biosensor (Fridley et al., 2014).

(Insert Figure 5 Here)

Both the 2DPN and the 2DPN card lateral flow biosensors showed improved analytical sensitivities, however, the assay time increased (5-10 min for normal lateral flow biosensor, 20-60 min for 2DPN) (Fridley et al., 2014; Fu et al., 2010a; Fu et al., 2011; Fu et al., 2012). As stated earlier, because the capillary flow time is proportional to the exponential of the distance, the greater the distance the longer the flow time to the end. However, two-dimensional lateral flow structures are useful for streamlining the signal amplification steps because they allow the incorporation of

multistep processes into one device while still retaining the positive aspects of conventional lateral flow biosensors.

3.4. Conceptual multiplexity – logic gate-based lateral flow biosensor

The multiplex expansion described above is mainly restricted by the space required to accommodate multiple test lines/dots. Therefore, expansion of multiplexing capacity via this approach can only ever be linear. However, the adoption of binary encoding concepts from computational science can enable exponential multiplex expansion. In addition, computational logic has been demonstrated within the molecular interactions themselves. This revolutionises the lateral flow biosensor to contain elementary decision making capabilities within the device itself, reducing the need for external computational analysis subsequent to the results display. In all of these cases, each analyte in a lateral flow detection system is assigned as an input (coded as "1", or no input ("0")). Multiple inputs involve various permutations and combinations depending on the computational logic embedded in the device, thereby resulting in multiplex detections.

The first demonstration of this proof-of-concept on a lateral flow biosensor was reported by Chen et al. (Chen et al., 2012). Their system employed "OR" (1,0; 0,1) and "AND" (1,1) logic gates for the successful detection of protein (thrombin) and small molecules (adenosine triphosphate, ATP) using aptamer recognition (Fig. 6) (Chen et al., 2012). The results showed excellent selectivity for analyte detection manipulated by the binary encoding concept. Subsequently, Huang et al. (Huang et al., 2014) successfully employed the "INH" logic gate (together with the "OR" logic gate) on a lateral flow biosensor. So far, "AND", "OR" and "INH" logic gates have been developed, which demonstrate the precise control binary encoding and the integrating of multiple inputs enables a definitive output. This paves the way for applying more advanced logic systems (e.g. "XOR" and "NOR" or even fully-featured automata (Lai et al., 2014; Poje et al., 2014; Wang and Katz, 2010)) to a lateral flow detection system.

(Insert Figure 6 Here)

In a slightly different approach, Li and Macdonald combined the binary encoding concept and lateral flow microarray to develop the first multiplexed digital-like result output as a 7-segment display on a lateral flow biosensor (Fig. 7) (Li and Macdonald, 2016). This lateral flow biosensor can detect seven analytes simultaneously on a linear scale, with potential to detect 127 different analytes using only seven label pairs. Although these lateral flow biosensors only allow the differentiation of discrete analytes, such a multiplex detection strategy is novel and beneficial for developing compact POC diagnostics when time and space are at a premium.

(Insert Figure 7 Here)

4. Issues that affect multiplexing analytical sensitivity and specificity

Regardless of the many improvements in diagnostic efficiency, the development of multiplexed lateral flow biosensors is still compromised by sensitivity and specificity. In most cases, the multiplexed lateral flow detection sensitivity is lower than that of the individual single-plex detections. This is mainly due to the specificity of the recognition molecules, as cross-reaction(s) occur among a mixture of analytes. The best way to solve this problem is to strictly select the recognition molecules for lack of cross-reactivity (Li and Macdonald, 2016). For example, application of monoclonal antibodies is more specific than the polyclonal antibodies (Lipman et al., 2005). Alternatively, replacement of antibodies with molecularly imprinted polymers (MIPs) (Bedwell and Whitcombe, 2015; Kandimalla and Ju, 2004), aptamers (Chen and Yang, 2015) or antibody mimetics (Baloch et al., 2016) is a more specific approach, as these synthetic molecules are tailor-made for detecting the specific analytes.

Other reasons for the compromised sensitivity and specificities in multiplexed lateral flow detection systems are associated with a range of issues: (1) the interference (suppression of binding) between multiple analytes and recognition molecules; (2) the sample is somewhat “diluted” due to the enlargement of the device dimension, especially in the multi-directional lateral flow biosensors (see Section 3.1.6.); (3) different binding kinetics among the recognition molecules; the position arrangement of spotting these molecules may alleviate this problem (Li and Macdonald, 2016); (4) buffer system compatibility in the multi-parameter lateral flow detection (Asanov et

al., 2012) (see Section 3.2.); and (5) certain reagent(s) in the sample buffer may cause "ghost band" which results in "false positives" (Cordray and Richards-Kortum, 2015).

In spite of the issues mentioned above, multiplexed lateral flow biosensors have been demonstrated to discriminate between closely related microbial or viral pathogens (Peng et al., 2016; Yang et al., 2015; Yonekita et al., 2013) and co-diagnosis of multiple-diseases (Corstjens et al., 2007). Multiplex lateral flow is particularly beneficial for biomarker screening as an alternative microarray format, as it alleviates several washing steps that are required for on chip-based microarrays, and has a lower implementation cost. (Rao et al., 2005; Taranova et al., 2013). Some multiplexed lateral flow biosensors even achieved ultra-low analytical sensitivities (e.g. femtomole) using novel tracers (signal transducing molecule) (Cary et al., 2011; Swanson and D'Andrea, 2013). In addition, many well-developed signal amplification methods (e.g. enzymatic or silver signal amplification) for single-plex lateral flow detection can be readily applied for the multiplexed lateral flow detection to enhance the analytical sensitivity. Taken together, the analytical sensitivity and specificity issues of multiplex lateral flow detection can be overcome with judicious selections of recognition molecules, tracers and signal amplification processes.

5. Summary and conclusions

Multiplexing diagnostics development has been driven by the need to validate a large number of disease biomarkers for healthcare. The development of multiplexed lateral flow biosensors offers a cost-effective way of improving efficiency, enhancing precision and reducing the assay time of POC diagnostics. In this review, we summarised all the current multiplexing approaches and assessed their advantages and disadvantages.

- The accommodation of multiple lines or dots in a single array is the most direct approach and requires no or limited modifications of the single-plex lateral flow biosensor. However, the number of lines and dots is limited by the space and Washburn theory.

- The lateral flow microarray format allows high-density detection but external equipment is needed for signal detection if the dots are too small to be seen, or if there are too many for a user to correctly interpret.
- The multi-parameter strategy is beneficial for diagnosis and treatment of genetic diseases. However, the design of such detection system requires careful consideration of cross-reactivity and differences in reaction kinetics between each detection parameter.
- The spatial multiplexity by 2DPN allows the sequential delivery of reagents towards one reaction zone with only single step activation. This strategy is particularly useful for signal amplification to enhance the sensitivity of the lateral flow biosensor, but has a drawback of increased assay time.
- The application of the binary encoding concept can expand multiplex detection exponentially for differentiation of discrete analytes, and even molecular logic can be embedded within a dot for molecular computational control of outputs.

While the development of multiplexed lateral flow biosensors is valuable, the device are still subject to the sensitivity and specificity inherent in traditional lateral flow devices, which can limit their application for clinical situations with enhanced sensitivity is critically required. However, there are many diverse applications where traditional lateral flow biosensors are providing exceptional reliability and flexibility for POC testing, such as the influenza lateral flow biosensors developed in quick responses to the outbreaks for H5N1 influenza virus (Soliman et al., 2010) and swine influenza virus (Ge et al., 2013), CardioDetect® FABP Rapid Test (VASOCARE Co., 2015) for verifying acute myocardial infarction, DPP® HIV 1/2 Assay (Inc., 2006-2014) for rapid diagnosis of HIV 1 and HIV 2 and NephroCheck® (Astute Medical, 2015) for detecting acute kidney injury biomarkers. The application of multiplex analyte detection for these situations can significantly improve efficiency of testing in terms of both cost and time, and promises to be an exceptional methodology improvement for advanced decision-making at the POC.

6. Future perspectives

The multiplex version of the lateral flow biosensor is emerging as the next wave of efficient and cost-effective diagnostics, and its development potential cannot be underestimated. These devices provide a platform for rapid translation of disease-biomarker detection technologies onto a simple and portable device that is suitable for non-skilled personnel to operate and interpret the diagnostic result. The applicability of the technology could be expanded by multiplexing at the level of multiple chemical processes (e.g. sample purification, concentration and detection), which could improve the reach of both the single and multiplex devices. In this regards, the harness of liquid flow merely via capillary force remains one of the biggest challenges (such as in sample preparation). There are a few demonstrations of integrating lateral flow detection with sample extraction and amplification using centrifugal microfluidics (Kim et al., 2014; Lee et al., 2015a) or with sample amplification and dilution as a self-contained device (Cordray and Richards-Kortum, 2015). In these cases, the lateral flow biosensors act as a syncretic part of a semi- or total analysis diagnostic system rather than merely a standalone detection procedure. Future research could therefore involve developing specific substrate or substrate treatments to assist the liquid flow manipulation. The immobilisation of effective recognition molecules that can capture and release the analyte(s) in a controlled manner would also be beneficial, particularly for auxiliary sample preparation integration.

Another consideration for implementation of multiplexing in lateral flow biosensors is a system for results interpretation, as end-users may struggle to correctly interpret read-outs when multiple lines appear on a device. Effective integration with technology that can autonomously provide user-friendly and intuitive interpretations will be critical for reducing interpretation errors. In this regards, many researchers are focusing on the development of electronic readers, which have the added advantage of enabling fluorescent or luminescent detection that improves the sensitivity of the device. However, the incorporation of multiple electronic read-out sensors in a multiplexing system would add significantly to the cost of the assay, reducing appeal particularly in middle to low-income countries. In this regards, we note our own solution that provides molecular encoding strategies that enable intuitive colourimetric readouts without requiring an external reading device (Li and Macdonald, 2016). Further integration with molecular computing operations would

thus be highly advantageous in developing autonomous and semi-intelligent lateral flow biosensing systems.

Multiplexing lateral flow technologies, particularly those truly accessible to the end-user, are extremely relevant for development of personalised diagnostics. Tailoring multiplex devices to monitor an individual patient's biomarker profile could radically improve health outcomes, particularly for patients with multi-syndrome ailments where cross-reacting drugs can reduce efficacy of treatments. However, this ideal implementation scenario would require significant improvements of processes surrounding device manufacture and testing. For example, a multiplex device currently does not offer any efficiency improvements in device testing and regulatory approval compared to manufacturing and testing several separate single-plex system, and may in fact complicate and delay development and approvals. This presumably explains why the majority of commercial multiplex devices to date are only those that operate as multiple parallel systems. The path forward, and a major focus of research in our own group, could be an approach that uses a generic platform technology that enables plug-and-play customisation from a collection of pre-approved individual biomarker assays.

Ultimately, the biggest driver for continued research in multiplex lateral flow biosensor technology is the radical improvements to healthcare that could result from such technology. These include dramatic improvements to customer diagnostic experiences, as well as corresponding reductions in healthcare budgets from improved diagnostic quality and efficiency. Multiplexed lateral flow biosensors embody precision, efficiency and user-friendliness. Because of this, we expect more diverse multiplexing solutions to continue to emerge, such that multiplex lateral flow biosensors become a significant part in the global diagnostic market within the next five to ten years.

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Fig. 1. Configuration and operation of a lateral flow biosensor. A: Construction of a lateral flow biosensor. A typical lateral flow biosensor consists of a membrane and several functional pads, which are connected and assembled in an order as sample pad, conjugate pad, membrane and absorbent pad, on an adhesive backing card. The conjugate pad is pre-deposited with anti-analyte antibody/tracer conjugates; the test line is pre-deposited with another anti-analyte antibody that binds to a different epitope to the analyte, and the control line is pre-deposited with a species-specific antibody that binds to the anti-analyte antibody conjugated to the tracer. B: Mechanism of a typical lateral flow biosensor. (i) Once an analyte (in solution) is loaded to the sample pad, the analyte will start to move along the lateral flow biosensor via capillary force. (ii) The analyte will be captured by the capture antibody/tracer conjugates when it comes across the conjugate pad. (iii) The resulting complex will be detected by the detection antibody when it reaches the test line, the excess capture antibody/tracer conjugates will keep on moving and be detected at the control line. All the unreacted substances will be absorbed by the absorbent pad. (iv) At the end, the appearance of two coloured lines indicates a positive result; a coloured control line indicates a negative result; and no coloured lines indicates an invalid result. The control line serves to validate the assay, which is independent of the presence of the analyte. C: Overview of different multiplexed lateral flow biosensors classifications.

Fig. 2. Different versions of multiplex lateral flow biosensors for identifying multiple analytes on a single device. A: Single lateral flow biosensor with many lines or dots in one array. B: Single lateral flow biosensor with multi-coloured lines. C: Single lateral flow biosensor with multiple dots as arrays. D: Multiple lateral flow biosensors with single line – parallelism. (Left): BD™ Directigen™ EZ Flu A+B kit (Becton, 2014). Courtesy and © Becton, Dickinson and Company. Reprinted with permission. (Right): RAID 8 and RAID TOX (Technologies, 2014). Courtesy of Alexeter Technologies. E: Bi-directional lateral flow biosensor. F: Multi-directional lateral flow biosensor with single line or dot. (i): Multiplexed bioactive paper sensor for heavy metals detections. Adapted with permission from Ref. (Hossain and Brennan, 2011). Copyright 2011, American Chemical Society. (ii) A tree-shaped device for detection of protein with self-calibration. From Ref. (Wang et al., 2010). Copyright

2010, Elsevier B.V.. (iii) Multi-directional lateral flow biosensor for multiplex whole cell bacteria analysis. From Ref. (Li et al., 2011). Copyright 2011, Elsevier B.V..

Fig. 3. Multi-parameter lateral flow biosensors. A: Construction of a multi-parameter lateral flow biosensor described by Oku et al. (2001). Their device involved the simultaneous detection of two analytes, Treponema pallidum (TP) antibody and Type B hepatitis (HB) surface antigen, which are both based on antigen-antibody interactions. The TP antibody and HB antigen were captured by tracer/TP antigen (tracer/antigen 2) and tracer/anti-HB antibody (tracer/anti-antigen antibody 1) conjugates respectively, and were subsequently detected by TP antigen/oligonucleotide (antigen 2/oligonucleotide 4) and oligonucleotide/anti-HB antibody (oligonucleotide 3/anti-antigen antibody 2) in the antibody test line and antigen test line via oligonucleotide 2 and oligonucleotide 1, respectively. B: Construction of a multi-parameter lateral flow biosensor described by Mao et al. (2014). Their device involved the simultaneous detection of two analytes, nucleic acid (60-mer DNA) and a protein (rabbit IgG), which the former is detected based on antigen-antibody interaction and latter is detected based on oligonucleotides hybridisations. The DNA and the protein were captured by the tracer/oligonucleotide 1 and tracer/anti-protein antibody 1 respectively, and were subsequently detected by oligonucleotide 2/biotin/streptavidin and anti-protein antibody 2 in the DNA test line and protein test line, respectively. Two control lines were also involved, one DNA control line (using oligonucleotide 3/biotin/streptavidin detecting excess tracer/oligonucleotide 1) and a protein control line (using anti-protein antibody 3 detecting excess tracer/anti-protein antibody 1) served to validate the test.

Fig. 4. Signal amplification in 2DPN and 2DPN card systems. A: Demonstration of signal amplification using a simple 3 inlets 2DPN. A spot of BSA-biotin was located upstream of the wicking pad and reagents were applied approximately simultaneously to the source pads using a multi-channel pipette in a single activation step. The images correspond to the approximate times of 1 s, 5 s, 25 s, 2 min, 5.5 min and 20 min, respectively. Streptavidin labelled gold (SA-G) reached the capture spot first, next a phosphate-buffered saline (PBS) wash and finally a gold enhancement (GE) solution. Note that the capture spot to the left of the wicking pad becomes

substantially darker by 20 min. From Ref. (Fu et al., 2010a). Copyright 2010, Elsevier B.V.. B: Easy-to-use 2DPN card format demonstrated for an amplified immunoassay. (Top) Conjugate, buffer and the gold enhancement reagent components were stored dry on the card for rehydration at the time of use. The user steps were (1) add sample to the pad labelled "S" (about 10 μL), (2) add water to the two pads labelled "W" (about 10 μL to the smaller one and about 40 μL to the larger one) and (3) fold the device closed to activate the multiple flows. (Bottom) 2DPN assay results for an analyte concentration of 200 ng/ml. The left panel shows the original pink signal after 8 min due to formation of a conventional sandwich structure with a GNP label in the detection region. The right panel shows the amplified, significantly darkened purple signal after 30 min. Adapted with permission from Ref. (Fu et al., 2012). Copyright 2012, American Chemical Society.

Fig. 5. Gold signal development and enhancement in an inkjet printed 2DNP system. (i) Device prior to fluid addition. Anti-PfHRP2 IgM was immobilised as a capture line, while goat anti-mouse IgG was used as a control line. Thirty μL of a mock sample consisting of PfHRP2 spiked at 100 ng/mL into fetal bovine serum was applied to the right-most inlet, where anti-PfHRP2 IgG-gold conjugate was patterned for rehydration. Immediately afterward, 40 and 100 μL of PBS were applied to the middle and left inlets, respectively. (ii) The initial gold signal appears 15 min after fluid addition. At the test line, the capture IgM, PfHRP2 antigen and gold conjugate form a "sandwich", while the control line consists of goat anti-mouse IgG binding to the mouse IgGs of the gold conjugate. (iii) Finally, the three gold enhancement solutions patterned on the left-most leg recombine upon rehydration and deposit more gold ions onto the surface of the GNPs, shifting the absorbance and turning the lines a dark black colour. Adapted with permission from Ref. (Fridley et al., 2014). Copyright 2014, American Chemical Society.

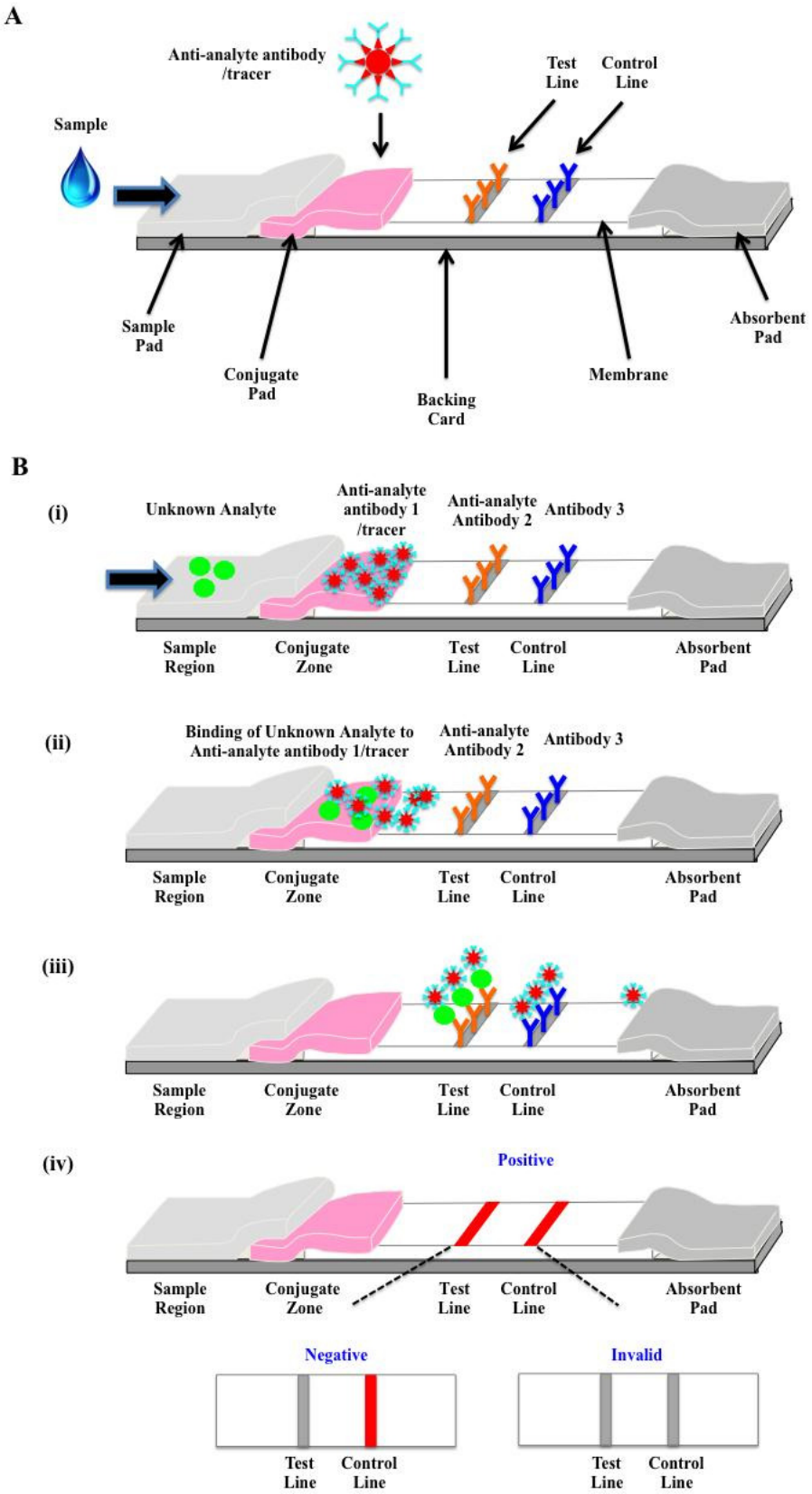
Fig. 6. Logic gate-based lateral flow biosensors using thrombin and ATP as inputs. A: Illustration of the design strategy of the "OR" logic gate and its corresponding lateral flow detection results. B: Illustration of the design strategy of the "AND" logic gate and its corresponding lateral flow detection results. In both cases, SA = streptavidin, CZ = the control zone and TZ = test zone. Adapted with permission from Ref. (Chen et al., 2012). Copyright 2012, American Chemical Society.

Fig. 7. 7-segment display of numbers on lateral flow biosensor. Addition of labelled nucleic acids mixtures resulted in the successful appearance of numbers (0 to 9). Adapted with permission from Ref. (Li and Macdonald, 2016). Copyright 2015, American Chemical Society.

Highlights of the review:

- Multiple analyte detection on paper-fluidic devices
- Multi-parameter lateral flow detection
- Spatial multiplexity by 2D paper network
- Conceptual multiplexity by logic gate-based lateral flow biosensor
- Sensitivity and specificity of multiplexed lateral flow detection
- Future perspectives of multiplexed lateral flow biosensors

Figure 1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Anti-analyte antibody /tracer
Test Line
Control Line
Sample
Sample Pad
Conjugate Pad
Backing Card
Membrane
Absorbent Pad
B
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Unknown Analyte
Anti-analyte antibody 1 /tracer
Anti-analyte Antibody 2
Antibody 3
Sample Region
Conjugate Zone
Test Line
Control Line
Absorbent Pad
(ii)
Binding of Unknown Analyte to Anti-analyte antibody 1/tracer
Anti-analyte Antibody 2
Antibody 3
Sample Region
Conjugate Zone
Test Line
Control Line
Absorbent Pad
(iii)
Sample Region
Conjugate Zone
Test Line
Control Line
Absorbent Pad
(iv)
Positive
Sample Region
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Test Line
Control Line
Absorbent Pad
Negative
Test Line
Control Line
Invalid
Test Line
Control Line

C

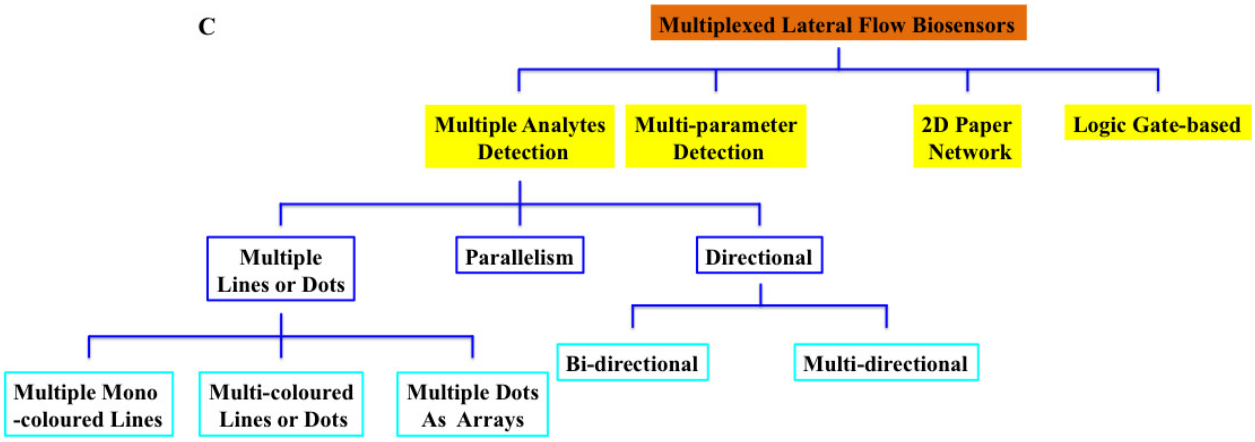


Figure 2

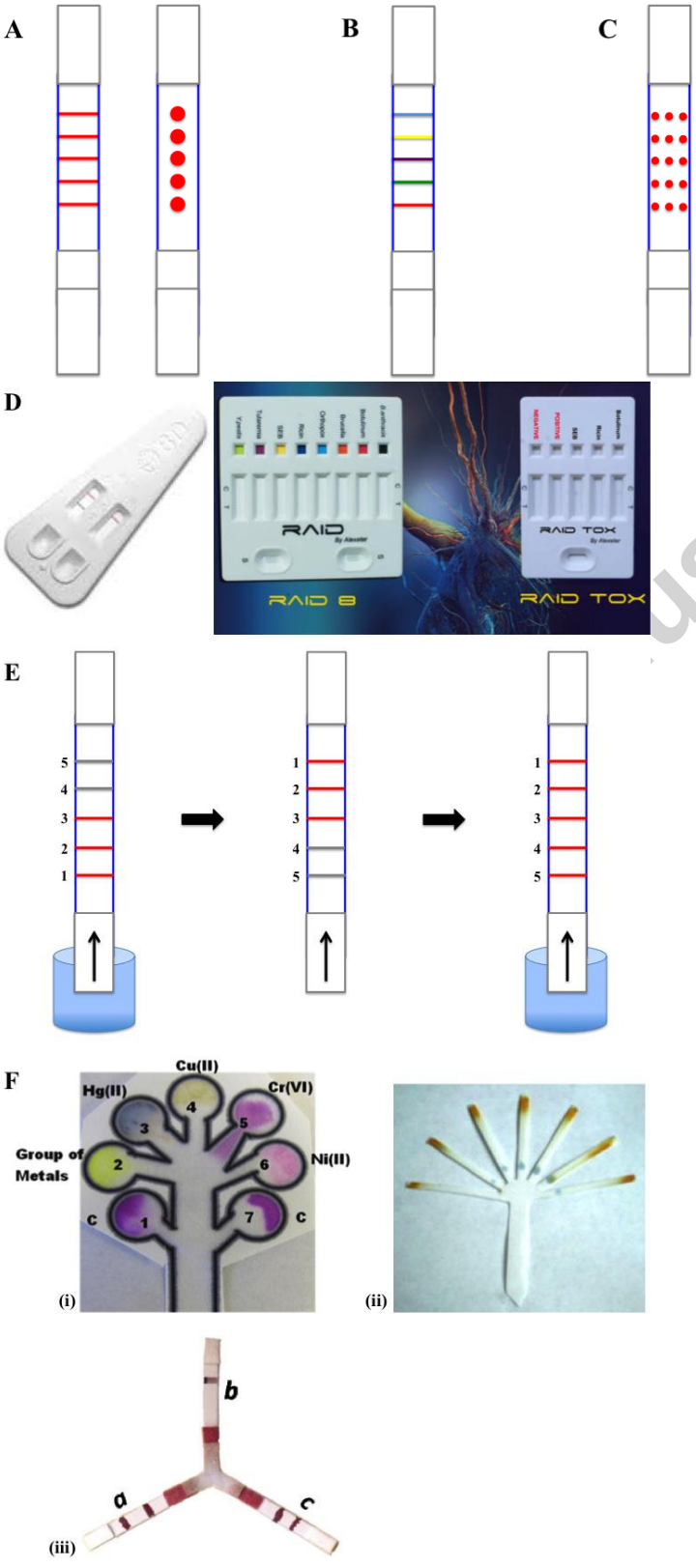
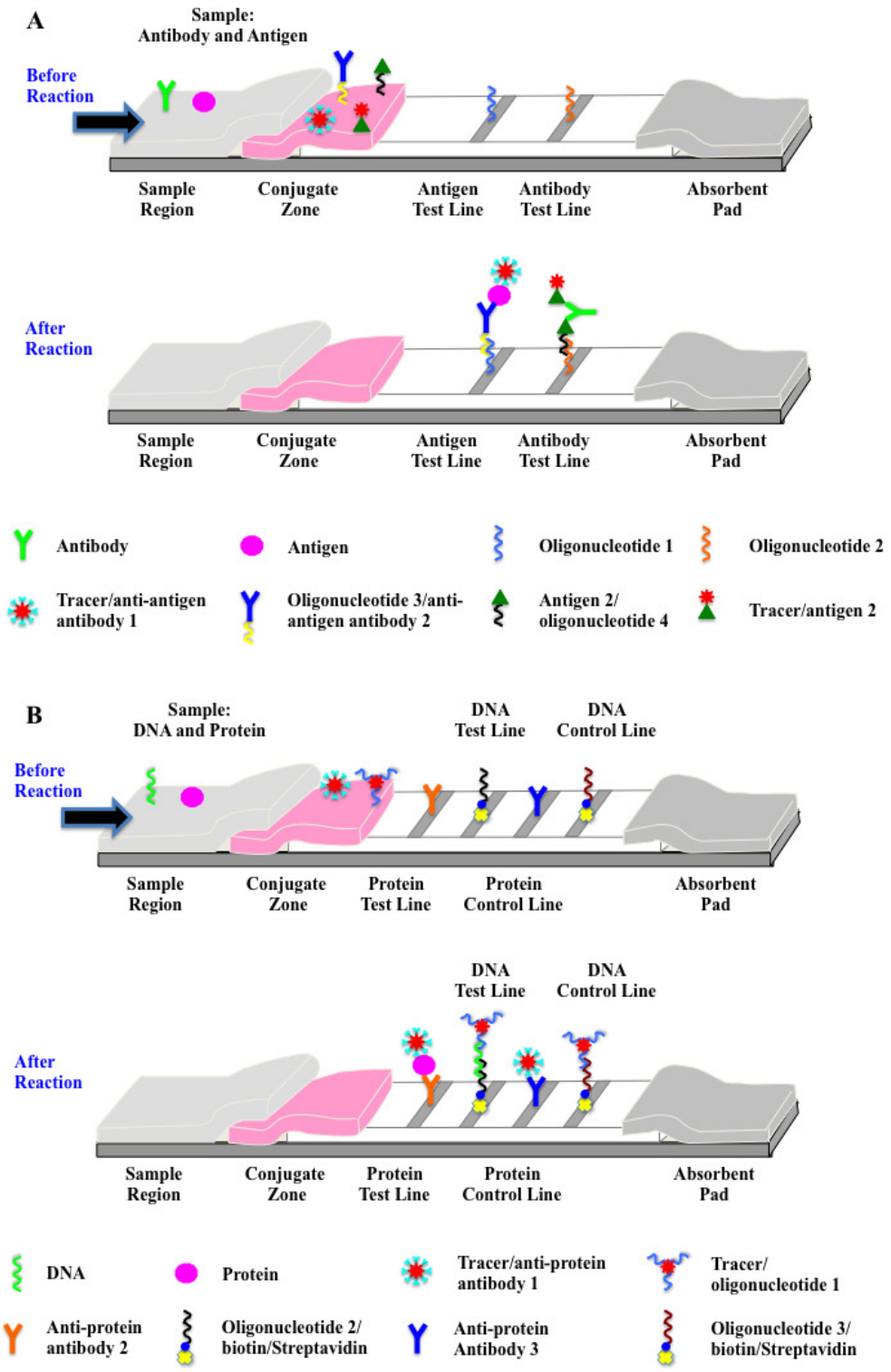


Figure 3


A
Sample:
Antibody and Antigen
Before Reaction
Sample Region
Conjugate Zone
Antigen Test Line
Antibody Test Line
Absorbent Pad
After Reaction
Sample Region
Conjugate Zone
Antigen Test Line
Antibody Test Line
Absorbent Pad
Antibody
Antigen
Oligonucleotide 1
Oligonucleotide 2
Tracer/anti-antigen antibody 1
Oligonucleotide 3/anti-antigen antibody 2
Antigen 2/ oligonucleotide 4
Tracer/antigen 2
B
Sample:
DNA and Protein
DNA Test Line
DNA Control Line
Before Reaction
Sample Region
Conjugate Zone
Protein Test Line
Protein Control Line
Absorbent Pad
DNA Test Line
DNA Control Line
After Reaction
Sample Region
Conjugate Zone
Protein Test Line
Protein Control Line
Absorbent Pad
DNA
Protein
Tracer/anti-protein antibody 1
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Oligonucleotide 2/ biotin/Streptavidin
Anti-protein Antibody 3
Oligonucleotide 3/ biotin/Streptavidin

Figure 4

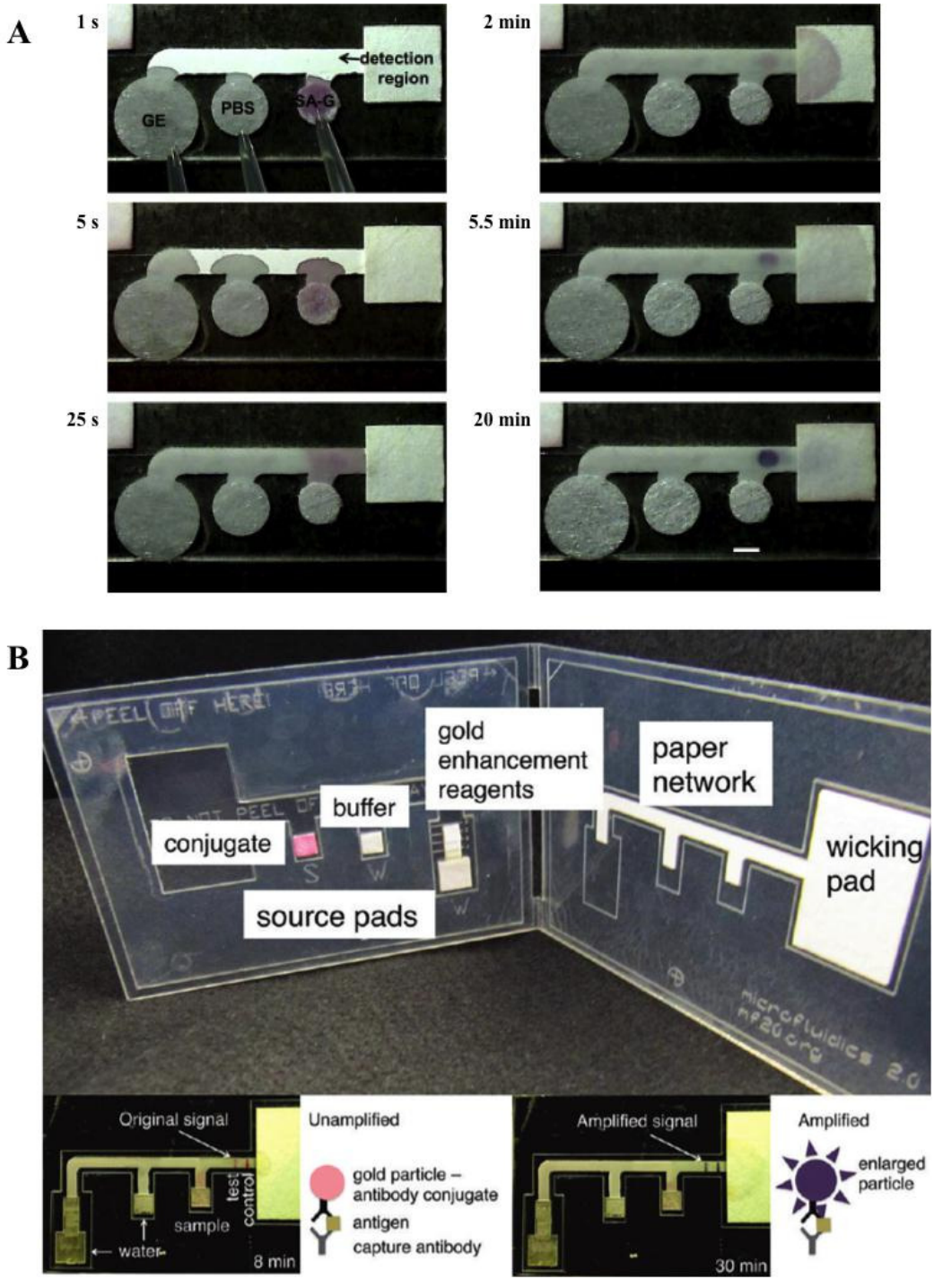
A
1 s
detection region
GE
PBS
SA-G
2 min
5 s
5.5 min
25 s
20 min
B
gold enhancement reagents
paper network
buffer
conjugate
wicking pad
source pads
Original signal
Unamplified
test
control
sample
water
8 min
gold particle – antibody conjugate
antigen
capture antibody
Amplified signal
Amplified
enlarged particle
30 min

Figure 5

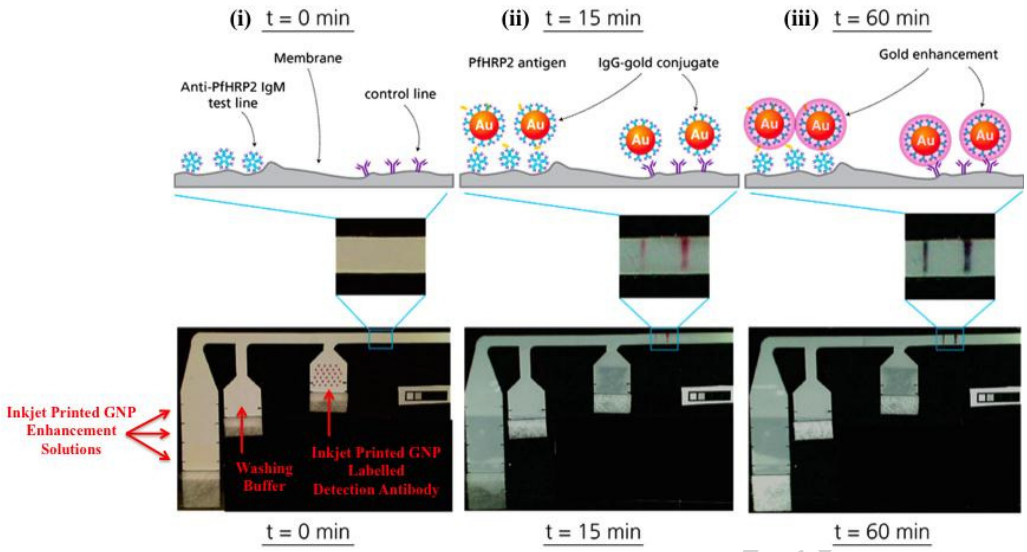

Figure 6

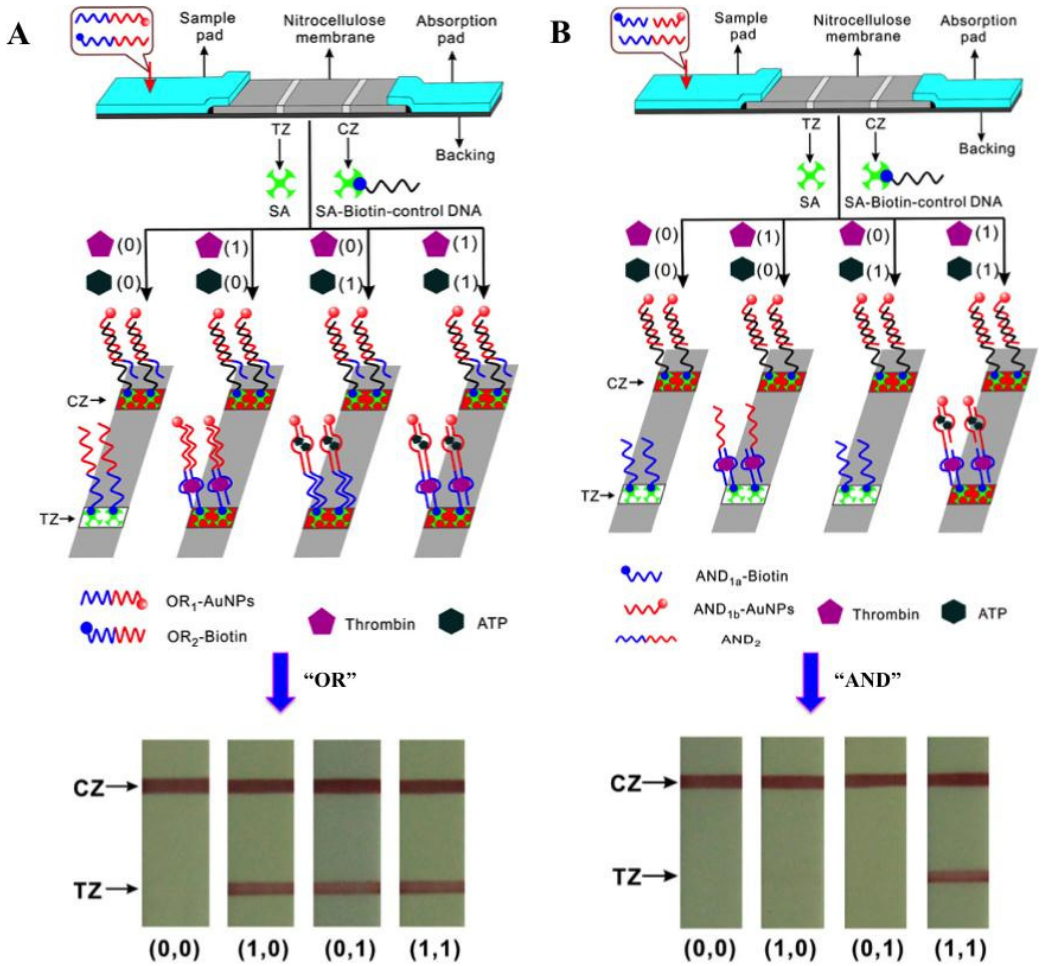

Figure 7