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ddRFC: A Scalable Multiplexed Droplet Digital Nucleic Acid Amplification Test Platform

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Abstract

Digital nucleic acid amplification tests (digital NAATs) have emerged as a popular tool for nucleic acid detection due to their high sensitivity and specificity. Most current digital NAAT platforms, however, are limited to a “one-color-one-target” approach wherein each target is encoded with a specific fluorescently-labeled probe for single-plex fluorometric detection. This approach is difficult to multiplex due to spectral overlap between any additional fluorophores, and multiplexability of digital NAATs has therefore been limited. As a means to scale multiplexability, we have developed a multiplexed digital NAAT platform, termed Droplet Digital Ratiometric Fluorescence Coding (ddRFC), via a padlock probe-based nucleic acid detection assay which encodes each nucleic acid target with a unique combination of 2 fluorophores. We detect this encoded two-color fluorescence signature of each target by performing digital amplification in microfluidic droplets. To demonstrate the utility of our platform, we have synthesized 6 distinct padlock probes, each rendering a unique two-color fluorescence signature to a nucleic acid target representing a clinically important sexually transmitted infection (STI). We proceed to demonstrate broad-based, two-plex, four-plex, and six-plex detection of the STI targets with single-molecule resolution. Our design offers a cost-effective approach to scale up multiplexability by simply tuning the number of molecular beacon binding sites on the padlock probe without

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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redesigning amplification primers or fluorescent molecular beacons. With further development, our platform has the potential to enable highly multiplexed detection of nucleic acid targets, with potentially unrestricted multiplexability, and serve as a diagnostic tool for many more diseases in the future.

Keywords

Picoliter droplet; digital nucleic acid amplification test; multiplexed nucleic acid detection; padlock probe; fluorescence spectroscopy

1. Introduction

Digital nucleic acid amplification tests (digital NAATs), which are used to amplify single copies of nucleic acid templates confined in hundreds to millions of miniaturized (fL - μ L) partitions, has emerged as a popular approach for nucleic acid detection, quantification, and characterization (Hindson et al., 2013; Ottesen, Elizabeth et al., 2006; Rane et al., 2015; Vogelstein and Kinzler, 1999). By leveraging powerful microfluidic microwell (Morrison et al., 2006; Shen et al., 2010), microchamber (Fan and Quake, 2007; Ottesen, Elizabeth et al., 2006; Warren et al., 2006), or microdroplet platforms (Hindson et al., 2011; Rane et al., 2015), most digital NAATs consume extremely low reagent volume, and by parallelizing amplification across all single-molecules within a sample, they achieve quantification of target analytes. Most importantly, digitization of target nucleic acid molecules in miniaturized reaction compartments (i.e., microwells or droplets) drastically increases the local concentration of the target, while simultaneously decreasing the effect of reaction inhibitors and amplification competitors, enhancing detection sensitivity and specificity when compared to traditional bulk-based NAATs (Hindson et al., 2011). As a result of these attributes, digital NAATs have seen increasing utility in various fields including pathogen identification (Athamanolap et al., 2019; Schoepp et al., 2016), genetic disorder screening (Lo et al., 2007; Tan et al., 2019) and cancer diagnostics (O'Keefe et al., 2018; Taly et al., 2013).

Unfortunately, most current digital NAAT platforms have been inherently limited by their low multiplexability (Dobnik et al., 2016; Whale et al., 2016). In most digital platforms, distinct nucleic acid targets within a sample are differentiated from each other via target-specific probes labeled with fluorophores of different colors. This “one-color-one-target” paradigm restricts their multiplexability to the number of fluorescence channels available in the detection platform. In order to avoid spectral overlap, and minimize cost and complexity, most commercially available fluorescent detectors for digital NAATs (*e.g.*, BioRad ddPCR™ (Hindson et al., 2011), QuantStudio™ (Thermo Fisher website) are typically limited to measurements in 2 colors (El Harrak et al., 2011; Hindson et al., 2011). Nonetheless, motivated by clinical need, an increasing number of diagnostic applications require higher order multiplexed detection. For example, in the field of infectious diseases, the ability to concurrently detect multiple target nucleic acids is critical for accurate diagnosis, especially in the case of polymicrobial infections, for effective management of antimicrobial therapy and drug resistance monitoring (Peters et al., 2012; Stacy et al., 2016).

Towards this end, researchers have sought to overcome the “one-color-one-target” paradigm by encoding target nucleic acids with fluorescence intensities instead of fluorescence color (Didelot et al., 2013; Dobnik et al., 2016; Taly et al., 2013; Zhong et al., 2011). While such a scheme may work within the confines of existing 2-color detectors and offer theoretically unlimited multiplexability, intensity-based encoding requires careful tuning of multiple primer pairs and primer/probe concentrations, a practically difficult task which necessitates carefully balancing the amplification efficiency, probe specificity, and competitive kinetics. Furthermore, scaling up such a scheme necessitates excessive amounts of primers and probes, becoming quickly cost-prohibitive. Ultimately the complexity of intensity-based multiplexing schemes has restricted its wide adoption (Whale et al., 2016). Therefore, there remains an unmet need for the development of a multiplexed digital NAAT platform that can be easily implemented for different DNA targets without cumbersome assay optimization.

In response, we have developed a multiplexed digital NAAT platform, termed Droplet Digital Ratiometric Fluorescence Coding (ddRFC). In this platform, we use target-specific molecular padlock probes that specifically bind to, and circularize upon binding with, nucleic acid target (Nilsson et al., 1994). Each unique combination of circularized padlock probe-target hybrid is designed to contain a specific ratio of binding sites for two distinctly labeled fluorogenic molecular beacons that generate fluorescence (red or green) upon binding to complementary target sequences (Zhang et al., 2018). This unique design results in each target being detected with a specific ratio of red to green (hereafter, R/G) fluorescence, allowing for unrestricted multiplexability in overcoming the ‘one-color-one-target’ paradigm. For simultaneous detection of multiple targets in a single reaction, each circularized padlock probe is encapsulated and amplified in picoliter droplets (hereafter, picodroplets) via hyperbranched rolling circle amplification (HRCA) (Lizardi et al., 1998) to facilitate two-color coincidence fluorescence detection of target amplicons within droplets. Importantly, this method facilitates easily scalable and cost-effective multiplexed digital nucleic acid detection. Using this simple design rule and detection approach, one can expand the degree of multiplexing by simply tuning the number of molecular beacon binding sites in the padlock probe design, while keeping the design of HRCA primers and molecular beacons unchanged regardless of the size of the target panel and the sequence for each of the targets.

To demonstrate our approach and platform, we detect specific genetic sequences from six major sexually transmitted infections (STIs) - namely, *Mycoplasma genitalium* (MG), *Herpes simplex virus* (HSV), *Neisseria gonorrhoeae* (NG), *Human immunodeficiency virus* (HIV), *Treponema pallidum* (TP) and *Chlamydia trachomatis* (CT). STIs present a particularly important case of infectious disease that demand sensitive and multiplexed diagnostic strategies in order to improve patient care and overcome their undue economic burden (Bristow et al., 2016; Gliddon et al., 2017; Kalou et al., 2016). The ddRFC scheme is exemplified by six-plex detection using only two molecular beacons of distinct fluorophores, indicating that the platform offers the ready means for multiplexed digital NAAT and a potential diagnostic tool for STIs and other infectious diseases.

2. Experimental

Our ddRFC starts with the mixture of 6 linear padlock probes and DNA targets, in which the corresponding linear padlock probes hybridize onto the targets and are ligated into circular probes at 55°C for 1 hour. The circularized probes are then mixed with HRCA primers and molecular beacons before being co-encapsulated into ~3pL droplets via a 10-μm flow-focusing junction on a polydimethylsiloxane (PDMS) microfluidic device. The droplets containing HRCA reaction mixture were incubated at 60 °C for 2 hours for digital amplification, followed by molecular beacon hybridization at 37 °C for 20 mins. These droplets are then reinjected into the detection module on the device. Red and green fluorescence intensities of each droplet are measured through a custom-built confocal microscope system, and the resultant R/G fluorescence ratios are used to identify the DNA targets after data processing using MATLAB. (Experimental details are provided as S.1 in Supplementary Material.)

3. Results and Discussion

3.1. Overview of the ddRFC platform

In realizing ddRFC, we combined the concepts of padlock-probe based fluorescence encoding of nucleic acids and microfluidic droplet-based digitization to achieve multiplexed, single-molecule detection of medically important nucleic acid targets. Our assay strategy relies on target-specific padlock probes (Sp et al., 2006) that hybridize to target nucleic acids and are detected via HRCA, an increasingly popular isothermal nucleic acid amplification method (Mohsen and Kool, 2016). In our assay, each linear padlock probe is designed to consist of three main regions, a target-complementary region at the 5' and 3' ends which facilitates hybridization to target nucleic acid sequences, a customizable fluorescence coding region which consists of a unique number of binding sites for green (AlexaF488) and red (AlexaF555) molecular beacon probes, and a universal HRCA primer binding site (Fig. 1A). In this work, we used this architecture to design 6 distinct linear padlock probes, each with a unique target-complementary region and unique number of green and red binding sites, resulting in a unique R/G ratio (0:1, 1:4, 1:1, 2:1, 4:1, and 1:0) for each targeted STI-causing species respectively (MG, HSV, NG, HIV, TP, CT) (Fig. 1B). These padlock probes share the exact same binding region for the HRCA primer pair, as well as the same sequences for red and green molecular beacon binding sites (Table S1), which allows us to use one pair of HRCA primers and the same set of molecular beacons for all the reactions (Table S2). In order to detect the presence and quantity of one or more of the aforementioned STI-causing species, we combine all six padlock probes along with DNA from an STI sample in a tube. In the presence of nucleic acid targets whose sequence is complementary to the target-complementary region, the initially linear padlock probes hybridize onto their corresponding target and are ligated into circular probes, while the remaining unbound linear padlock probes are digested by exonucleases. These circularized probes are then co-encapsulated into picodroplets along with HRCA primers as well as red and green molecular beacons, such that each picodroplet contains either a single copy of the circular padlock probe or no circular padlock probe, as governed by the Poisson distribution (Hindson et al., 2011; Vogelstein and Kinzler, 1999) in order to enable digital amplification. The picodroplets are

all collected and incubated together to facilitate digital HRCA, where within picodroplets containing circular probes, primers elongate along the circles and generate DNA templates with multiple tandem repeats with the same sequences as the initial linear padlock probes. The amplified DNA templates then serve as hybridization sites for red and green molecular beacons and produce specific R/G fluorescence ratios in picodroplets, which corresponds to the designed target-specific R/G ratio for each padlock probe. By measuring the red and green fluorescence intensities of each picodroplet using a two-color laser-induced fluorescence (LIF) system and calculating its R/G fluorescence ratio, our platform is capable of decoding and identifying all the DNA targets that were present in the original sample (Fig. 1C). In this manner, our ddRFC platform not only surpasses the ‘one-color-one-target’ paradigm by encoding nucleic acid targets with distinct fluorescence ratios, but also achieves cost-effective multiplexed digital nucleic acid detection using simply one pair of universal primers and two molecular beacons.

3.2. Broad-based detection of STI-causing pathogens using ddRFC

Settling on a modular microfluidic device (Fig. S1) (Kaushik et al., 2017; Mach et al., 2019; Zhang et al., 2019) interfaced with a two-color LIF instrument (Fig.S2) (Kaushik et al., 2017; Rane et al., 2012; Zhang et al., 2019), we have demonstrated the broad-based detection of STIs using our ddRFC. We first synthesized 6 oligonucleotides derived from conserved gene sequences of MG, HSV, NG, HIV, TP and CT as the nucleic acid targets (Table S3) for these experiments. We then tested each synthetic oligonucleotide by combining 3.75 pM of the synthetic targets into a mixture of 50 pM of the 6 linear padlock probes. From the resultant fluorescence time trace of droplets for each DNA target, the vast majority of picodroplets display baseline background fluorescence, indicative of empty “negative” droplets, whereas a small fraction of picodroplets display higher fluorescence signals, indicative of target-amplified “positive” droplets (Fig. 2A).

The relative intensities between positive red and green droplets correlated well with the designed R/G ratio for each target and can therefore be used to differentiate each STI target in our platform. For example, in the case of MG target DNA, the linear padlock probes were designed to have an R/G ratio of 0:1. From the picodroplet fluorescence time trace, we likewise do not observe any positive red picodroplets and several positive green picodroplets, resulting in a measured R/G positive droplet intensity ratio of 0. For the HSV, NG, HIV, and TP DNA targets, we observe coincident positive picodroplets in both red and green detection channels. Here, for designed R/G ratios of 1:4, 1:1, 2:1, and 4:1, we measure linearly increasing R/G ratios of 0.76, 1.71, 3.67 and 6.89 respectively. We note that the while the measured R/G ratios are distinguishable and follow the linear trend of the designed R/G ratios, they do not fully match the designed R/G ratios due in part to the difference between output power of two excitation laser sources, as well as the configuration of the optical components in our LIF detector which may not fully match the spectra of the red and green fluorophores. Finally, for the CT sample, which had a designed R/G ratio of 1:0, we only observe positive droplets in the red channel, resulting in a measured R/G ratio of infinity. To demonstrate that these 6 pathogens can be unambiguously distinguished, we plotted the measured red and green fluorescence intensities of at least 1000 positive picodroplets in each sample in a two-dimensional scatterplot. We have observed 6 well-

separated populations with distinct slopes which indicated distinct R/G ratios resultant from these six samples (Fig. 2B). The average R/G ratio and experimental variation was determined for each target and plotted, revealing excellent separation and negligible crosstalk between measured R/G ratios and high linear correlation between designed and measured R/G ratios ($R^2 = 0.9973$) (Fig. 2C). We note that for each individual species, from 79.0% to 99.4% picodroplets (Fig. S3) were decoded to the accurate target species while a small portion of them fell into adjacent populations. These rare picodroplets with R/G ratios overlapping with adjacent populations results from the variation in detected fluorescence signals, which may be caused by the incomplete hybridization between molecular beacons and amplified templates due to steric hindrance caused by coiled HRCA amplicons (Jarvis et al., 2006; Lizardi et al., 1998), the variance in amplification efficiency of in-droplet HRCA reaction, the optical configuration of the LIF detector as well as picodroplet size variation. Nevertheless, the t-test we performed between adjacent populations have further demonstrated that the average R/G ratios for each population are significantly different from each other (Table S4), indicating excellent separation between measured ratios. Therefore, the crosstalk should have very limited effect on identifying the STI species present in the sample (Göransson et al., 2009). In addition to synthetic targets, we have also verified the performance of ddRFC for detecting more clinically-relevant genomic DNA (Fig. S4).

3.3. Two-plex detection and quantification of STI-causing pathogens using ddRFC

To demonstrate the utility of ddRFC for multiplexed NAAT, we first performed simultaneous detection of 2 STI targets. We chose to work with NG and HIV synthetic DNA targets, as both of these targets have been assigned intermediate R/G ratios (1:1 and 2:1) that are among the closest to each other from the 6 designed R/G ratios, and therefore represent the most challenging targets to demux. These 2 DNA targets were combined with a mixture of the 6 linear padlock probes for ligation and then co-encapsulated with molecular beacons and HRCA reagents into picodroplets, followed by incubation and LIF detection. The two-dimensional scatterplots of red and green fluorescence intensities from at least 1000 picodroplets (green versus red) was plotted (Fig. 3A), showing that each of the 2 targets (orange for HIV and yellow for NG) can be qualitatively differentiated from one another. Furthermore, the measured R/G ratios (1.59 for NG and 3.26 for HIV) correspond well with the broad-based, single-plex experiments, further validating our ddRFC multiplexing methodology. Taken together, ddRFC can not only identify one potential target out of multiple possible candidates, but also can be used to simultaneously detect multiple targets in a single reaction, with single-molecule resolution.

Digital detection of nucleic acids using ddRFC also provides us the ability to quantify STI targets, which is important for monitoring the pathogen load, diagnosing the status of an infection and guiding appropriate antimicrobial treatment (Bissessor et al., 2015; Saag et al., 2018; Shen et al., 2011). To assess in-droplet quantification, we performed 2-plex detection HIV and NG DNA, but varied the input copy number of each target relative to the other (HIV:NG = 1:25, 1:5, 1:1 and 5:1 respectively). For each sample tested, we measured 2 noticeably distinct R/G ratios, indicative of the 2 distinct STI targets. In order to demonstrate quantification, we plotted the R/G ratios across all positive picodroplets in each experiment on a histogram, which clearly showed the 2 subpopulations of HIV and NG target-

containing picodroplets (Fig. S5). Each subpopulation in each histogram was quantified and the relative quantity of HIV to NG picodroplets was determined for each experiment. Plotted against the expected ratio of HIV to NG DNA, the measured quantities show a highly linear correlation ($R^2=0.9995$) and negligible variation ($n=3$) across multiple experiments (**Fig. 3B**). We have noted that the detected ratios of HIV to NG DNA are not the same as the expected ratios, which is probably caused by the variation in amplification and/or ligation efficiency of padlock probes due to their different lengths and/or sequences. The detected ratios of HIV to NG DNA are lower than those expected, suggesting relatively lower amplification efficiency of HIV padlock probe compared with NG padlock probe.

3.4. ddRFC for higher order multiplexed detection

Our ddRFC methodology is amenable to a high degree of multiplexing, limited only by the number of unique linear padlock probes. To maximize the multiplexability in this work, we demonstrate simultaneous detection of 4 and 6 STI targets in the same reaction. Here we prepared 2 separate tubes – one containing NG, HIV, CT, and MG DNA, and another tube containing all 6 of our DNA targets – and proceeded with the linear probe hybridization, ligation, droplet HRCA, and LIF detection. The resulting scatter plots from the four-plex (Fig. 3C), and six-plex (Fig. 3D) reactions, show clearly separated STI pathogen-containing picodroplets, as well as distinct R/G ratios.

4. Conclusion

We have developed a novel platform, ddRFC, for multiplexed digital nucleic acid detection by encoding each nucleic acid target with a specific fluorometric ratio in picodroplets. Comparing to conventional ‘one-color-one target’ coding schemes, our platform assigns a pre-designed fluorescence ratio to each target through padlock probe chemistry, thus eliminating the issue of spectral overlap and significantly expanding the multiplexability of our detection platform. Our linear padlock probes are designed to share common molecular beacons and primer binding sequences for different targets, enabling a scalable and economical approach for multiplexed digital detection of nucleic acids. We demonstrate the utility of our platform by achieving up to 6-plex digital detection of 6 STI pathogens using only two standard fluorophores. With this simple design rule and detection approach, we can easily expand the target panel by simply tuning the number of molecular beacon binding sites on the padlock probes to generate more target-specific R/G fluorescent ratios, while keeping the universal primers and common set of molecular beacons for all the targets.

Our ddRFC provides a simple solution to higher-order multiplexing for digital NAATs and can be readily coupled with existing two-color fluorescence detection platforms. Furthermore, the ddRFC strategy need not be limited to droplet microfluidic platforms and can be applied to other digital NAAT platforms, including microwells and microchambers, providing a readily adaptable solution to an expanding user base. In addition, the multiplexability of our ddRFC can be readily expanded by designing more molecular beacon binding sites, though the practical multiplexing capacity might be limited by the potential overlap between R/G ratios that are too close to each other. Therefore, we note that further expansion of the multiplexability may focus on incorporating more fluorescence colors or

replacing HRCA with other nucleic acid amplification assay such as PCR. With these improvements and its demonstrated robust performance, we foresee ddRFC as a potentially strong digital NAAT platform for large-scale multiplexed detection of nucleic acids and even potential diagnostic screening of STIs in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A multiplexed digital NAAT platform (ddRFC) is developed which encodes each nucleic acid target with a unique fluorescence ratio
- ddRFC surpasses the ‘one-color-one-target’ paradigm
- ddRFC achieves cost-effective multiplexed digital nucleic acid detection using simply one pair of universal primers and two molecular beacons

Fig. 1. Overview of ddRFC platform.

(A) Our linear padlock probes consist of three main regions: a target complementary region at two ends, a customizable fluorescence coding region consisting of a target-specific number of binding sites for red and green molecular beacons, and a universal HRCA primers binding site. In the presence of corresponding nucleic acid target, the originally linear padlock probe hybridizes onto the target and is ligated into a circular probe. (B) In this work six padlock probes are designed and encoded with distinct ratios of binding sites for red and green molecular beacons to target conserved sequences from 6 most common STI pathogens respectively. (C) Schematic illustration of our ddRFC from padlock probe ligation and co-encapsulation, to digital amplification and LIF detection.

Fig. 2. ddRFC for broad-based detection of STI-causing pathogens.

(A) The red and green fluorescence time traces of picodroplets for each DNA target are recorded using our LIF system. The vast majority of picodroplets display baseline background, indicating that they are empty “negative” droplets. Within “positive” droplets displaying significantly higher fluorescence signal, the relative intensities between red and green fluorescence intensities correlate well with the designed R/G ratio for each DNA target. A clear increasing trend of R/G fluorescence ratio has been observed in the picodroplets resultant from the DNA targets with increasing pre-designed R/G ratios. (B) The two-dimensional scatterplot for red and green fluorescence intensities of 6 samples, of which each contains one synthetic target respectively, have shown 6 well-separated populations with distinct slopes, indicating that picodroplets from these 6 samples have produced 6 distinct R/G fluorescence ratios. (C) The average measured R/G ratios have shown high linear correlation with designed R/G ratios with relatively small error bars from over 1000 positive droplets in each sample.

Fig. 3. ddRFC for multiplexed nucleic acid detection.

(A) A sample containing both NG and HIV synthetic targets has been tested using our ddRFC platform. The resultant two-dimensional scatter plot for red and green fluorescence intensities of over 1000 positive droplets have shown two clearly distinguishable populations, yellow for NG and orange for HIV. (B) The measured ratios of input copy number of HIV to NG targets has shown a high linear correlation with the expected ratios ($R^2=0.9995$), with small variation across multiple experiments ($n=3$). (C) The two-dimensional scatterplot for a sample containing four DNA targets (NG, HIV, CT, and MG) has shown four well-separated populations. The populations locating on x axis (red fluorescence only) and y axis (green fluorescence only) respectively indicate the presence of both CT targets (designed $R/G = \infty$) and MG targets (designed $R/G = 0$), meanwhile 2 other populations have shown the similar distribution as those in 2-plex detection. (D) The two-dimensional scatter for a sample containing all 6 DNA targets has shown 6 well-separated populations, demonstrating our ddRFC platform for multiplexed nucleic acid detection with distinct R/G ratios.