MEGAPIXEL DIGITAL PCR
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ABSTRACT
We report a microfluidic ‘megapixel’ digital polymerase chain reaction (PCR) device that uses a surface tension–based sample partitioning approach along with integrated dehydration control to enable high-fidelity single DNA molecule amplification in 1,000,000 reactors of picoliter volume, in arrays with densities up to 440,000 reactors cm\(^{-2}\). This device achieves a dynamic range of 10\(^7\), single-nucleotide-variant detection below one copy per 100,000 wild-type sequences, and the discrimination of a 1% difference in chromosome copy number.

KEYWORDS: Digital PCR, microfluidics, DNA, single molecule, high throughput, genomics, diagnostics.

INTRODUCTION
Advances in basic research and molecular diagnostics capabilities are intimately coupled to the development of ever more precise and sensitive measurement technologies. Quantitative (q)PCR is the current gold standard for DNA measurement and genetic diagnostics but remains poorly suited to measurements of absolute concentration, has a limited precision (~20%), and presents difficulties in reliably detecting low-copy-number templates due to nonspecific amplification and competitive side reactions. qPCR measurements are thus inadequate for demanding applications such as early detection of cancer and monitoring of residual disease[1], analysis of single-cell gene expression[2], and the diagnosis of fetal genetic disorders using small allelic imbalances in circulating DNA.

THEORY
A promising solution to such measurement problems is digital PCR[3], a single-molecule counting technique. Digital PCR works by partitioning a sample at limiting dilution followed by PCR amplification and endpoint detection to identify the presence or absence of template molecules in each reaction. All performance metrics of digital PCR, including sensitivity, precision and dynamic range, improve with the total number of digital reactions performed. Assuming a random distribution of single molecules and reliable detection of single molecules, it may be shown that the expected response, precision, and dynamic range of digital PCR are respectively given by (1), (2) and (3):

\[ N\lambda = N \ln \left( \frac{N}{N - x} \right) \]
\[ \Delta \lambda / \lambda = 4.6 \lambda^{-1} N^{-1/2} \left( e^{1/4} - 1 \right)^{1/2} \]
\[ DR = N \ln(N/10) \]

Where, N is the number of chambers, \( \lambda \) is the average number of molecules per chamber, \( x \) is the number positive chambers, \( N_\lambda \) is the best estimate of copy number, \( \Delta \lambda / \lambda \) is the fractional precision, and DR denotes the dynamic range of measurement. Assay density, scale and reduced volume are thus critical to the development of next-generation digital PCR systems. Microfluidics offer attractive platforms for digital PCR by providing reduced reaction volumes, increased throughput, higher single-molecule detection efficiency, reduced contamination, and reduced cost.

EXPERIMENTAL
Megapixel microfluidic devices having 1,000,000 digital reaction chambers (Figure 1a) were fabricated using multilayer soft lithography[4] with modifications to allow for the inclusion of an embedded parylene C layer (~2µm) to prevent excessive dehydration (Figure 1b). Reaction components, including PCR master mix, probes, primers, and templates were assembled and mixed off chip prior to analysis in microfluidic digital PCR arrays. First, solutions were dead-end loaded into the de-
vice at 12 psi with the exit port of the array blocked by the actuation of integrated microvalves (25 psi). Following complete filling, FC40 oil (3M) was injected into the main flow channel at 14 psi in order to partition the samples (Figure 1c). After PCR thermocycling, the devices were scanned at 0.5 micron per pixel (Figure 1d) and analyzed using a custom image analysis software written in C for automated segmentation of chambers (Figure 1e).

**RESULTS AND DISCUSSION**

Here we present a valve-free microfluidic digital PCR device that performs a million single molecule PCR reactions in uniform arrays of pl volume chambers having densities up to 440,000 reactions cm$^{-2}$ (Figure 1a). Our device uses surface tension to achieve partitioning of the sample into a uniform array of pl volume sub-reactions. The sample is injected into a poly(dimethylsiloxane) (PDMS) device featuring a bifurcating channel network, having a cross-section of 3 µm x 3 µm, that connects to linear arrays of 10 pl (20 µm x 20 µm x 25 µm) “dead-end” chambers. The reagent solutions are then pushed in the device until complete filling and partitioning of the chambers is achieved by flushing an immiscible fluorinated oil that preferentially wets the channel walls, displacing the remaining aqueous phase (Figure 1c). Partitioning of the array is complete in approximately 1 minute. Although the gas-permeability of the device material is needed for dead-end loading, this property also leads to water vapor transport and rapid evaporation during thermocycling when using small volumes. To resolve these competing requirements, we developed a fabrication process to embed a ~2 µm thick layer of low permeability polymer (parylene C) above the digital PCR array, creating a permeation barrier (Figure 1d). In addition, water vapor gradients at the periphery of the array are controlled by the inclusion of hydration lines (100 µm x 100 µm) which fix the vapor pressure and enable robust single DNA molecule amplification and unambiguous detection in pl volume reactors (Figure 1e).

**DYNAMIC RANGE, SENSITIVITY AND PRECISION**

A digital PCR array of 10$^6$ chambers provides a theoretical dynamic range of 7 logs. To experimentally establish the response of the Megapixel device we measured the abundance of a single copy gene (RPPH1, chromosome 14) over a 10-fold serial dilution of human genomic DNA spanning 6 orders of magnitude in concentrations from $3 \times 10^6$ to ~2.4 haploid genomes per 10 pl chamber (~920 fg/µL to ~780 ng/µL). The observed fraction of positive chambers ranged from 0.00028% to 90.8% and showed excellent agreement with the theoretical binomial response ($R^2=0.9978$). To further characterize the dy-
namic range at high fill factor, experiments using a synthetic fragment of the RPPH1 gene were performed over concentrations ranging from approximately $6 \times 10^6$ copies per chamber (1 fM) to 9.5 copies per chamber (1.6 pM). These were again in excellent agreement with the theoretical response ($R^2=0.9999$). At the highest concentrations tested we observed a fill-factor of 99.994% before reaching saturation, corresponding to an average of 9.7 molecules per chamber.

Next we tested the sensitivity of our device in detecting rare mutations, defined as the lowest measurable ratio of two target sequences differing by a single nucleotide variation (SNV ratio). Two-color digital PCR measurements of mixtures of plasmids containing the wildtype JAK2 kinase gene and a V617F variant were found to be accurate over relative dilutions ranging from 1:1 to 1:10,000 ($R^2=0.9993$). We note that the lowest relative concentration measured ($10^{-4}$) is comparable to the inherent error rate of Taq polymerase, and represents a fundamental limit for methods that use a pre-amplification step. However, polymerase errors in digital PCR without pre-amplification should result in the co-detection of both alleles. Thus we hypothesized that SNV detection at concentrations below the polymerase error rate would be possible by excluding double-positive chambers. To test this we first loaded a single plasmid into $10^6$ chambers at a concentration of 1.39 copies per chamber and detected a total of 38 SNV false positives with co-detection of both probes in 36 of these chambers (94%), an observation that cannot be explained by random co-localization ($p = 0.001$; binomial test). Errors that occur after the first two rounds of amplification are not detected so that, assuming an equal frequency of single base substitutions, we estimated the polymerase error rate to be between $2.6 \times 10^{-5}$ and $1.6 \times 10^{-4}$, which is in close agreement with previously reported values ($3 \times 10^{-5} - 1.1 \times 10^{-4}$)[5]. We next loaded two 500,000 chamber sub-arrays at relative allele concentrations of 1:100,000 and 2:100,000 (concentration ~1 plasmid/chamber), and detected a total of 5 and 11 isolated SNV positive chambers. From this we determined the measured SNV ratio to be $2.7 \times 10^{-5}$ and $5.9 \times 10^{-5}$ respectively. This corresponds to a SNV detection limit of approximately 1:100,000.

An array of 1,000,000 chambers has the theoretical precision needed to discriminate a difference in relative concentration of 0.6% with 99% sensitivity and 99% specificity. Ten replicate measurements of normal human genomic DNA for two single copy genes, RPPH1 (chromosome 14) and HLCS (chromosome 21), yielded mean copy numbers of 425,885 (s.d. = 652.6) and 409,435 (s.d. = 639.9) respectively, with an average ratio of HLCS/RPPH1 of 1.040 (s.d. = 0.0027). We next evaluated the precision of our device in detecting small allelic imbalances by measuring the relative copy number of the HLCS and RPPH1 genes in normal human genomic DNA spiked with varying amounts of trisomy 21 (T21) genomic DNA ranging from 6% to 2%. Reliable discrimination of 2% and 3% enrichment was obtained reproducibly using sub-arrays of 100,000 chambers, while 1% enrichment was only well-resolved using full 1,000,000 chamber arrays.

CONCLUSION
In addition to enabling new measurements in biomedical research and diagnostics, the high assay density in our device has important implications for the adoption of digital PCR as a routine analytical tool. Thus, we contend that megapixel digital PCR or similar high-density formats will ultimately replace real-time qPCR as the standard analytical tool for absolute DNA measurement.

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