Facile profiling of molecular heterogeneity by microfluidic digital melt

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This work presents a digital microfluidic platform called HYPER-Melt (high-density profiling and enumeration by melt) for highly parallelized copy-by-copy DNA molecular profiling. HYPER-Melt provides a facile means of detecting and assessing sequence variations of thousands of individual DNA molecules through digitization in a nanowell microchip array, allowing amplification and interrogation of individual template molecules by detecting HRM fluorescence changes due to sequence-dependent denaturation. As a model application, HYPER-Melt is used here for the detection and assessment of intermolecular heterogeneity of DNA methylation within the promoters of classical tumor suppressor genes. The capabilities of this platform are validated through serial dilutions of mixed epialleles, with demonstrated detection limits as low as 1 methylated variant in 2 million unmethylated templates (0.00005%) of a classic tumor suppressor gene, CDKN2A (p14ARF). The clinical potential of the platform is demonstrated using a digital assay for NDRG4, a tumor suppressor gene that is commonly methylated in colorectal cancer, in liquid biopsies of healthy and colorectal cancer patients. Overall, the platform provides the depth of information, simplicity of use, and single-molecule sensitivity necessary for rapid assessment of intermolecular variation contributing to genetic and epigenetic heterogeneity for challenging applications in embryogenesis, carcinogenesis, and rare biomarker detection.

INTRODUCTION

Variability in gene regulation is a fundamental characteristic of biology, allowing cellular adaptation in many states such as development, stress response, and survival. One prominent manifestation of this variability with respect to human health is Darwinian selection within human disease (1–3). From bacterial infections to cancer, diseased populations rely on the ability to generate different phenotypes to respond and thrive in their environment (4–7). Hence, many diagnostic principles rely upon the detection of genetic and epigenetic biomarkers, which often consist of minor DNA variations that occur even down to the single-nucleotide level, such as mutations, deletions, frameshifts, and methylation, that provide selective advantage (8–10). Likewise, in many cases, early detection and diagnosis largely improve the patient’s prognosis and survivability (11). Although the advent of so-called “digital” analysis technologies has furthered the detection of rare modifications in a binary manner, the stochastic and variable nature of disease onset and progression could be better understood with a more multidimensional analysis approach. Thus, a system that enables both rare detection and population profiling on a molecule-by-molecule basis could greatly aid in detection and understanding of disease.

Molecular analysis has been significantly advanced by techniques such as next-generation sequencing and digital polymerase chain reaction (dPCR) approaches, including droplet digital PCR (ddPCR), but currently available instrumentation has significant drawbacks that impede its applicability for detection and assessment of heterogeneity within rare molecular variants. Although sequencing can provide genome-wide sequence information, its limited sensitivity, complex workflow, and high cost undermine its utility for routine detection of very rare biomarkers. In contrast, current dPCR technologies theoretically have single-copy sensitivity but are not readily adaptable to multidimensional analysis.

High-resolution melt (HRM) is a facile means of assessing sequence variations within a target locus by measuring the release of DNA-intercalating dye during temperature-dependent denaturation. Current commercial dPCR technologies, such as the QX200 (Bio-Rad) and Raindance Systems, have limitations preventing ready adaptation to HRM as a molecular profiling tool, while most noncommercial ddPCR technologies with real-time monitoring capabilities require droplet trapping before monitoring, severely compromising throughput (12, 13). Although a number of commercial array-based dPCR strategies do exist, namely, QuantStudio (Thermo Fisher Scientific) and Biomark (Fluidigm), they lack HRM analysis capabilities, likely due to numerous technical challenges. The limited number of in-house attempts has generally been encumbered by significant obstacles, such as maintaining thermal control and uniformity throughout the device (14), achieving sufficient sequence resolution of a few nucleotides (15), observing dim fluorescence and optical noise from small reaction volumes, and managing stochastic amplification variations from digital starting concentrations (16). These issues primarily stem from fundamental limitations in commercially available hardware or the use of off-the-shelf analysis software.

Here, we use a comprehensive bottom-up approach involving both hardware and software solutions aimed at addressing these technical challenges. We achieve this by design and fabrication of the HYPER-Melt platform, consisting of a 4096-nl well static array microfluidic device and optical-thermal platform for digital PCR and digital HRM. This high-density array can digitize and analyze thousands of individual molecules while limiting reagent consumption to that of a typical microtiter well, and provide an absolutely quantitative measure of sequence heterogeneity within a target locus throughout a molecular population. Our all-in-one chip design is simple to fabricate and use, offers a consolidated workflow, enables rapid and automatic loading and digitization, and is robust to reaction conditions. The
optical-thermal setup minimizes light scattering and thermal instability, and we introduce a postprocessing software solution that can be used to circumvent irregularities imposed by hardware limitations, thereby permitting reproducible HRM-curve acquisition.

Although DNA hypermethylation of tumor suppressor genes has been under investigation as a biomarker for early detection of many types of cancer for some time (10, 17, 18), the etiology and precise effects of heterogeneity within hypermethylation is an important phenomenon that has engendered great interest in the epigenetic community (9, 19, 20). We previously demonstrated the utility of a quasi-digital HRM-based technique called DREAMing for the detection and analysis of DNA methylation variability in liquid biopsies (21), which is often aberrant in cancer and can result in dysregulation of gene expression (22–24). These liquid biopsies are an attractive means of noninvasive monitoring and screening but typically only contain cell-free DNA (cfDNA) (<50 ng/ml) in the plasma (17), of which fractions of 0.01% or lower may be tumor-derived DNA (25). The ultrahigh sensitivity of HYPER-Melt qualifies the technique for noninvasive detection and monitoring of genetic and epigenetic variability assessment via liquid biopsy analysis.

While the DREAMing technique provides a means of evaluating methylation at single-copy sensitivity, the unavailability of commercial high-density, HRM-capable instrumentation restricts its use for many research and clinical applications. Here, we incorporate the DREAMing technique into the HYPER-Melt platform to achieve highly parallelized assessment of intermolecular methylation variation at single-copy sensitivity in two well-established methylation biomarkers for cancer, cyclin-dependent kinase inhibitor 2A (CDKN2A) and N-myc downstream-regulated gene 4 (NDRG4). We then demonstrate the application of the HYPER-Melt platform to provide absolute assessment of methylation heterogeneity within the NDRG4 locus from cfDNA extracted from plasma of liquid biopsies of colorectal cancer (CRC) patients in comparison to healthy individuals. Overall, the HYPER-Melt platform allows for extremely sensitive quantification and analysis of methylation heterogeneity and provides multidimensional information about single molecules that could offer greater understanding of intermolecular variability with respect to biological processes such as carcinogenesis and development.

RESULTS
Overview of epiallelic profiling by HYPER-Melt
To achieve high-density molecular profiling, we developed a microfluidic chip and associated instrumentation for digitization and interrogation of individual DNA sequences (Fig. 1). The chip is first rapidly loaded with the reaction mix containing rare epiallele targets (Fig. 1A). The chip is then placed on a flatbed heater to perform PCR and HRM. A mirrorless interchangeable lens camera (MILC) acquires images of the entire chip at each temperature increment during the melt process (Fig. 1B). The images are processed and analyzed for each reaction chamber to procure a melt curve derivative from which the location of the peak defines the melt temperature (Fig. 1C). Finally, the melt temperatures of all the ampiclons were calculated to catalog the initial template methylation variants and compiled in a histogram for quantitative methylation heterogeneity analysis (Fig. 1D).

Assessment of DNA methylation heterogeneity follows the assay principles laid out in our previous report (21). Briefly, primers were developed such that they provide methylation-preferred amplification, in which all epiallelic variants of the target sequence are amplified, with a significant bias toward the amplification of the partially and fully methylated template molecules. This strategy greatly increases detection sensitivity in the presence of a high background of unmethylated DNA. After PCR, HRM analysis is performed by observing the release of a DNA-saturating dye (for example, EvaGreen) during temperature ramping (26). As the temperature is increased, the amplicons denature, resulting in a measurable decrease in fluorescence. The temperature at which exactly half of the template strands of a particular ampiclon become denatured is termed the melt temperature ($T_m$) for that target sequence. Following bisulfite conversion, methylated templates exhibit an increased $T_m$ due to the greater stability of C- versus T-base stacking (27).

In the digital methylation profiling assay, reaction volumes containing both unmethylated DNA and a single rare epiallele produce a melt curve with two peaks. The left-most peak pertains to the unmethylated/background DNA. The right-most peak corresponds to the rare epiallele, whose methylation density can then be resolved by its $T_m$. Because of the digitization of differentially methylated epialleles, HYPER-Melt provides absolute quantitation of all methylated variants and overall methylation heterogeneity.

The dynamic range of HYPER-Melt and other digital approaches can be described by the Poisson distribution: $P(n, \lambda) = (\frac{\lambda^n e^{-\lambda}}{n!})$, where the probability that any given chamber will contain $n$ copies depends on $\lambda$, the average concentration in copies per chamber volume. For the principle of HYPER-Melt to apply, each chamber may contain no more than one rare methylated variant. By definition, the dynamic range of the device scales with the total number of chambers available. Our proof-of-concept design contains 4096 wells per microfluidic chip, which facilitates detection and discrimination of more than 1500 heterogeneously methylated variants simultaneously, and can be readily scaled to higher quantities.

HYPER-Melt digital chip characterization
The digital melt chip was fabricated using soft lithography techniques for polydimethylsiloxane (PDMS) microfabrication. The microfluidic design uses a 4096-well static array, fabricated from only two standard materials, glass and PDMS (Fig. 2A).

A number of key design strategies were incorporated into the chip design to address some issues that commonly compromise the performance of PDMS-based devices for PCR-based assays. For example, soft lithography devices often experience sample loss during high-temperature reactions due to the permeability of PDMS. Previous investigators have incorporated additional materials as an impermeable boundary layer (28–30), which are effective but add to the complexity and duration of fabrication. In lieu of this approach, we sought to prevent evaporation without the need for specialized equipment or materials and without the addition of layers that may introduce noise in the fluorescence signal.

In our device, sample loss and optical scattering were mitigated by the development and use of an ultrathin fabrication technique (fig. S1) to reduce the external volume of PDMS. We aimed to fabricate a single pattern layer with <25 µm between the height of the pattern and the surface of the PDMS (Fig. 2B). However, PDMS membranes with a thickness of less than ~100 µm experience strong adhesion to a high-aspect ratio silicon mold and are likely to tear during removal, damaging both the mold and chip. Therefore, our technique uses a sacrificial PDMS layer to enable imprinted release of the 80-µm pattern layer from the mold without tearing and with high reproducibility. The incorporation of the ultrathin pattern layer, a glass coverslip, and
hydration lines effectively prevents evaporation during digital PCR (31), minimizes optical interference, and reduces thermal deviation, which is critical to digital melt analysis.

Samples were initially prepared by mixing the reagents and sample off-chip. The chip undergoes desiccation after fabrication to produce a negative pressure differential across the seal of the inlet (Fig. 2C). When punctured, the sample was drawn into the device by the force of the vacuum, filling all the wells in less than 5 s. Next, an oil-based solution is pressure-driven through the channels. Surface tension between the partitioning oil and the aqueous reaction mixture prevents the oil from entering the wells, thus isolating and digitizing the template molecules (movie S1), typically in less than 3 min. During PCR, the oil remains pressurized to prevent the sample from exiting into the channels. The addition of PDMS in the partitioning oil produces a solidified, permanent barrier between reaction chambers that is maintained throughout the assay. This technique allows the microfluidic chip to be easily transported for digital melt or other analyses without requiring continuous pressurization and minimizes contamination risk by locking the post-PCR samples in place.

**Thermal-optical integration**

To achieve a digital melt platform capable of high-throughput imaging of the high-density array, we required a solution that provided an adequate compromise between two, often competing, challenging imaging goals: real-time fluorescence monitoring of submicroliter volumes and rapid wide field-of-view acquisition. To reduce the effects of noise that can interfere with the melt signal, we developed an imaging system capable of measuring the fluorescent changes in nanoliter volumes of thousands of chambers simultaneously by adapting and optimizing both conventional and unconventional imaging components (fig. S2A). To increase the signal level and minimize the Poissonian fluctuation of the nanoliter reaction volumes, a multifaceted optimization approach was used by taking into account the Poissonian statistics and fluorescence efficiency of the excitation light and fluorescence emission, respectively (32). Excitation light intensity was increased via incorporation of multiple light-emitting diodes (LEDs) to provide strong and steady excitation, as well as uniform illumination of the sample (fig. S3). The illumination angle of 45° minimizes background noise from reflection of the excitation source. In observing fluorescence emission, the detection probability of the sensor depends on the sensor area and integration time. Therefore, to increase detection probability, we used a full-frame 36-mm × 24-mm sensor and optimized the image exposure, ISO, and aperture to balance the signal-to-background ratio with a reasonable image capture rate.

To perform on-chip heating, we devised a strategy to address local nonuniformities and thermal fluctuations that commonly occur in commercial flatbed heaters, which can greatly compromise data integrity in applications such as HRM requiring precise temperature actuation. Toward this end, we used multiple stabilizing heat block layers to equilibrate the thermal mass, comprising a flatbed adapter, thermal paste contact enhancer, and a silicon wafer to efficiently and uniformly transfer heat across its surface. The final stabilizing layer is the glass slide of the digital melt chip itself. Previous PCR attempts used thin glass slides (0.01 mm) that are rapidly conductive but lack rigidity, resulting in significant bowing during temperature ramping. Therefore, we substituted a thicker, more rigid glass slide (1 mm) to provide thermal dissipation, improve robustness of the chip handling,
and achieve more efficient fabrication. The additional thermal stabilization layers increased thermal uniformity of the chip by 172.3% (fig. S2B). The final thermal-optical module acquires melt curves in just under 10 min with a 0.2°C temperature resolution and a signal-to-noise ratio of 5.60 (fig. S2C).

**HYPER-Melt analysis**

To assess the intermolecular heterogeneity of the amplicons, we collected images of the entire chip during temperature ramping (Fig. 3). All pictures were aligned to the first image using an open-source automated image registration (AIR) program (33). Next, the pixel-space locations of each well were semiautomatically identified by a customized MATLAB program. The user selects the four corner points of the array image from which a script generates a linearly scaled mask of the array. Misalignment between the chip and camera detector is initially corrected by applying a homography transformation of the array mask using the four user-selected points and the generated corners. The well boundaries are then shrunk by 10% on each side so that only the central mass of the mixture is used for measurement. Finally, each well mask within the grid is locally optimized in a 5 × 5 pixel neighborhood to correct for any additional warping and to identify the center of each well (fig. S4).

Once the grid mask is defined, it is propagated throughout each image to obtain the fluorescence intensity values from each pixel at each time point. The outlying points from each well are removed, and the remaining intensity values from each frame within the temperature interval were averaged to obtain a raw curve. Several filters were tested to ascertain those that best preserved the integrity of the raw data. A combination of a low-pass filter and Savitz-Golay filter was found to best remove additional noise while still preserving melt information. Next, the derivative was calculated to find the temperature at the inflection point of the melt curve termed the “melt temperature” (T_m). A digital melt histogram was created that reveals the four distinct populations, which can be readily separated by simple thresholding. The methylation density of the original template in each reaction volume was then classified by its melt temperature, providing a quantitative analysis of methylation heterogeneity on a locus-specific, molecule-by-molecule basis.

**Performance evaluation: Synthetic targets**

To assess the capabilities of the HYPER-Melt platform, we used synthetic targets representative of various bisulfite-converted sequences.
of the tumor suppressor gene CDKN2A (p14ARF) as a model system. Four methylation densities were analyzed: 0, 33, 67, and 100%. The unmethylated (0%) sequence represented the background population and was set to 500 copies/nl. The three methylated variants were digitized on chip, amplified, and identified by HYPER-Melt. The detected number of targets for each epiallele was calculated by the number of positive melt-discriminated wells while accounting for a Poisson distribution.

In terms of sensitivity, the platform demonstrated absolute quantitation, as the calculated number of copies closely matched the expected number (Fig. 4). A serial dilution of methylated variants at concentrations of 0, 1, 10, 100, and 1000 copies per chip was performed in the presence of the 2 million unmethylated epialleles. The average number of copies detected represents the combined average of the three epiallelic variants detected and discriminated among the unmethylated background population in each experiment. Experiments for each of 0, 1, and 10 expected copies were repeated. A linear fit from ~1 to 1000 copies produces a slope of 0.91, suggesting that overall, 91% of the expected DNA was detected with an $R^2$ (coefficient of determination) value of 0.97. This discrepancy likely represents slight material deformation that deviates from the expected geometry used during calculations. The results of this analysis demonstrate that the HYPER-Melt platform provides robust, absolute quantitation over five orders of magnitude, attaining accurate HRM-based detection down to single-copy sensitivity.

The ability of HYPER-Melt to distinguish rare sequence variations was demonstrated through discrimination of epiallelic populations among the background of unmethylated epialleles for each serial dilution (Fig. 5). Representative traces of fluorescence signals from individual wells are shown for each test (Fig. 5B), which were classified by melt temperature via thresholding and color-coded by methylation density. Once discriminated by melt temperature, the digital result was converted to a methylation density heatmap (Fig. 5C), which was then further quantified and analyzed via a methylation heterogeneity histogram (Fig. 5D). The master mix was divided such that the same reaction volume was loaded into the chip as well as a control benchtop reaction for validation of the PCR and melt success (Fig. 5E).

Overall, the specificity of the HYPER-Melt platform for rare heterogeneously methylated epialleles was demonstrated down to fractions as low as 1 in 2 million unmethylated copies, or 0.00005%. Even higher specificities are ostensibly achievable by the HYPER-Melt platform, as it is only limited by the appropriate dynamic range of the application. Heterogeneously methylated epialleles can be readily differentiated with $<1^\circ C$ resolution, enabling sequence-specific discrimination of $<4$ CpG sites.

**Performance evaluation: Liquid biopsies**

Last, we sought to demonstrate the potential clinical utility of the platform by using HYPER-Melt for the analysis of methylation heterogeneity of a cfDNA biomarker of CRC from liquid biopsies of cancer-free volunteers compared with patients diagnosed with metastatic CRC (mCRC). We chose to use an alternate methylation biomarker, N-Myc downstream-regulated gene 4 (NDRG4), which has been implicated in neurite outgrowth and cellular differentiation via the regulation of transcription factors (34) and whose in-tissue clinical sensitivity for CRC is far greater (86%) (35, 36) than that of CDKN2A (p14ARF) (28%) (37). DREAMing primers for NDRG4 were designed and optimized to achieve single-copy sensitivity, as previously described (21).

cfDNA from the plasma of four cancer-positive patients and four apparently healthy volunteers was extracted, purified, and underwent bisulfite conversion, as detailed elsewhere (21). Each sample was amplified, melted, and analyzed using the same protocol as described above. For comparison, each sample was also assessed for NDRG4 methylation using quantitative methylation-specific PCR (qMSP) (38, 39). Of the four CRC-positive patients, three of four were positive for NDRG4 methylation by HYPER-Melt, only one of which showed clear positivity with the NDRG4 qMSP assay (Fig. 6). Of the four samples from healthy volunteers, one was positive for NDRG4 methylation by HYPER-Melt, albeit at very low levels of low to intermediate methylation.

From these data, it is readily apparent that HYPER-Melt exhibits both considerably higher analytical sensitivity when compared to qMSP. This is not unexpected, as MSP is designed to reliably only detect fully or very heavily methylated epialleles. In practice, however, most MSP-based assays can still amplify heterogeneously methylated epialleles, albeit at much lower sensitivity and efficiency. Hence, samples such as CRC1, which exhibit significant copy numbers of low- and medium-density methylation by HYPER-Melt, exhibit weak, nonquantitative positivity by qMSP (40). In all, HYPER-Melt demonstrated 20 to 300 times or more analytical sensitivity than the qMSP assay in all methylation-positive samples. The greater analytical sensitivity of HYPER-Melt resulted in detection of NDRG4 methylation in two additional CRC-positive patients (CRC1 and CRC2) that are negative or nearly negative by qMSP. While beneficial in the case of the CRC-positive patients, HYPER-Melt also detected methylation in one of the healthy controls. The presence of very low copy numbers of heterogeneously methylated NDRG4 epialleles in sample N1, a

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**Fig. 4. Detected versus expected DNA copy number.** Mixed epialleles were diluted into a background of 2 million unmethylated templates. After amplification and HRM-based discrimination, the detected copy number of each epiallele was calculated according to a Poisson distribution. The average epiallelic detected copy number was compared to the expected. The array chip demonstrates linearity from ~1 to 1000 copies. x refers to expected number of copies of epiallele (DNA), and y refers to detected number of copies.
50-year-old male, may be due to natural methylation heterogeneity resulting from early-stage epigenetic drift (41).

**DISCUSSION**

The HYPER-Melt platform provides a practical and rapid technique for absolutely quantitative assessment of intermolecular variability in DNA methylation levels of a target locus with a demonstrated sensitivity of 1 in 2 million background copies at <1°C resolution (~4 CpG sites). Its clinical applicability was demonstrated with commensurate performance, achieving 20 to 300 times more sensitivity than the gold standard (qMSP), for assessment of methylation heterogeneity of *NDRG4* in liquid biopsies. HYPER-Melt was also compared to more sensitive methods (that is, ddPCR), where it demonstrated more sensitive detection and discrimination of heterogeneous targets (fig. S9).

There are a number of key innovations that, when combined, enabled achievement of significantly higher HRM sensitivities than previously demonstrated in the literature.

The development of a wide field-of-view imaging platform is critical to achieving real-time fluorescence monitoring of such a large array of nanoliter reaction chambers. Present commercial digital PCR technologies suffer from a number of key drawbacks and are not yet suitable for digital HRM applications. Droplet-based technologies, such as the QX200 (Bio-Rad) and Raindance systems, rely on a single-point readout, which is not amenable for real-time monitoring of fluorescence intensity. QuantStudio 12K Flex (Thermo Fisher Scientific) and Biomark (Fluidigm) are not equipped for precise temperature control, and the high cost of their OpenArray (Thermo Fisher Scientific) and Digital Array IFC (Fluidigm) cartridges undermines their clinical attractiveness. Many of the reported real-time fluorescence tracking microfluidic chips have a limited number of chambers (42, 43), use complex imaging components (14), do not track volumes below tens of nanoliters, and do not demonstrate digital starting concentrations (42–44). Other microfabricated chip designs for digital PCR involve a complicated fabrication step to insert an evaporation barrier (28, 29) or use mechanically intensive microvalves for partitioning (28). Multilayer microvalve partitioning strategies suffer from complex fabrication methods and a limiting valve footprint that reduces scalability potential, as well as require intricate operational instrumentation. Large-array image acquisition techniques usually necessitate the use of a microarray scanner or similar instrumentation that includes motion and panning of either the camera or object of interest, followed by subsequent
image stitching or the use of multiple imaging setups to image the entire volume at sufficient resolution and sensitivity (28, 45, 46). Although these methods can be very effective at end-point detection, in real-time imaging, they accrue costs of time, photobleaching, and thermal instability, thus rendering these methods not suitable for a high-throughput digital melt platform.

In contrast, HYPER-Melt uses readily available imaging components, such as an LED array and a commercial MILC, a predominantly automated processing pipeline, and a simple and scalable valveless microfluidic design, to achieve small-volume, wide field-of-view imaging and high-fidelity melt curve acquisition. Because native current commercially available hardware for a thermal-optical platform is not sufficient to produce high-fidelity melt curves in a highly parallelized manner, we optimized the configuration of readily available components to minimize thermal-optical noise and implemented a software solution to extract parallelized single-molecule information. A common strategy for object detection in fluorescent images is thresholding; however, this is susceptible to error due to artifacts and requires very uniform illumination of the sample. Alternatively, we introduced a detection technique using a known-mask mapping. This technique capitalizes on the known
grid design and offers several advantages. First, a map generation script was created to allow for automatic generation based on a few geometric user inputs so that the overall analysis can be universally applied to any grid-based design. Furthermore, the localized well-search algorithm relaxes the requirements of illumination uniformity due to its restriction to the neighborhood of each well.

The presented microfluidic solution provides absolutely quantitative sequence heterogeneity information at ultrahigh sensitivity while maintaining the practicality and simplicity necessary for routine use. Notably, recent studies have demonstrated that even minor populations of mutated molecules or heterogeneously methylated epialleles can have direct clinical implications. For example, intermediate DNA methylation heterogeneity is predictive of metastatic versus localized clones in Ewing sarcoma (47), and observations of methylation reprogramming on a single CpG site basis in acute myeloid leukemia patients emphasize that molecular heterogeneity plays an important role in tumor progression and adaptation to the microenvironment (48). Adenomatous polyposis coli (APC) mutations at levels as low as 0.01% could be indicative of early CRC (49). HYPER-Melt provides an enabling platform for similar studies looking to examine the role of rare, heterogeneous genetic or epigenetic aberrations on a molecule-by-molecule basis without excessive cost and time.

In terms of potential clinical use, one of the highly touted advantages of liquid biopsies is the ability to ostensibly sample overall tumor heterogeneity using a simple blood draw. However, the extremely low copy numbers of circulating tumor DNA within the overall cfDNA samples make assessment of this heterogeneity extremely challenging. As a proof of concept, the potential power of our platform was demonstrated by using these liquid biopsy samples. Overall, the considerable dynamic range of the platform allowed ultrasensitive detection of heterogeneous NDRG4 epialleles down to 1 in 2 million unmethylated epialleles and in clinical plasma samples at concentrations of less than 150 copies per milliliter of plasma. HYPER-Melt also demonstrates robustness against multiple samples and targets, providing more information about cfDNA epiallelic fractions than either MSP or traditional DREAMing while achieving over 100-fold greater sensitivity.

The capabilities of the HYPER-Melt platform were demonstrated with the DREAMing assay (21), which is designed to detect methylation density of rare molecules among a background of unmethylated or perceived healthy molecules. The primers were designed to introduce an amplification bias toward the rare molecules, allowing single-copy detection even in a high background. This approach achieves highly sensitive, absolute quantitation of the digitized methylated single-copy detection even in a high background. The resolution density information realized by HYPER-Melt provides an all-in-one analysis platform that is able to detect methylated single-copy detection even in a high background. The ability to fully digitize samples with HYPER-Melt while maintaining a considerable dynamic range allows absolute quantification for all methylation patterns without bias, thereby enabling the assessment of hypomethylation in CpG-sparse loci, which commonly occurs during carcinogenesis (10, 22, 47, 54). Other applications of fully digital HRM include ultrasensitive detection of rare genetic/epigenetic variants, including mutation and single-nucleotide polymorphism detection (55–57). Furthermore, digital melt curve analysis also provides considerable advantages over digital PCR–based approaches by allowing facile verification of PCR products, which is a critical feature for avoiding false positives in rare target detection and diagnostics (58). Intermolecular variability is of great interest in other applications such as embryogenesis (59), carcinogenesis (7, 9, 60), and regulatory circuitry (6, 61), for which HYPER-Melt provides a practical and facile means of further investigation.

There are a few drawbacks in the current platform that should be noted. One fundamental issue with the microfluidic platform is the potential disparity between sample volume and reaction volume. While our chip was specifically designed to achieve compatibility with existing bisulfite conversion kits, larger samples would likely require incorporation of a concentration method, such as the previously described methylation-on-beads technique (62). The reported benefits of the platform are limited to examining a single target in a single sample, which restricts the clinical applications of this platform to those with a well-defined, highly specific locus of interest. Alternatively, multiplexing could be achieved by splitting samples into subarrays but would require the use of greater sample volumes or additional wells available for digitization to maintain assay performance metrics. Furthermore, the current chip is limited in throughput to the assessment of a single sample per run. Although eight samples can be run per day, each requires an individual chip, and thus the platform has relatively low sample throughput. However, this limitation is similar to many other digital real-time analysis or sequencing technologies in which the sensitivity can be increased by dedicating more of the reaction space to a single sample.

In conclusion, HYPER-Melt provides an all-in-one analysis platform of molecular profiling and heterogeneity analysis. The platform allows deep insight at the single-copy level of any target of interest. Overall, this platform has the potential to detect intermolecular variability at the single-copy level of any target of interest.
ultrahigh sensitivity even in difficult and highly heterogeneous samples, allowing more comprehensive investigation of the dynamics and stochasticity of DNA molecular heterogeneity. Furthermore, the practicality and high digitization power of the platform offers a tool for rapid and efficient DNA heterogeneity analysis by rapidly interrogating hundreds to thousands of individual molecules in parallel.

MATERIALS AND METHODS

Chip fabrication

Microfluidic chips were fabricated using standard photolithography and soft lithography techniques. To fabricate the reusable master mold, a silicon wafer was dehydrated, and oxygen plasma-treated (TechniCraft PE-IL) at 80 W for 1 min. SPR220-7 photoresist (MicroChem) was spun at 2600 rpm for 1 min and then soft-baked at 115°C for 20 min. The wafer was then exposed to the channel pattern using a mask aligner at 1150 J/cm² and developed in CD26. After a hard bake at 200°C for 6 hours, SU-8 3050 was spin-coated on the wafer at 1400 rpm for 1 min. After a soft bake at 95°C for 20 min, the wafer was aligned to the well array mask, exposed at 300 J/cm², developed, and baked at 200°C for 1 hour.

Each microfluidic chip was fabricated from this mold using soft lithography and our unique ultrathin ~80-μm layering technique (fig. S1). The wafer was first silanized to protect the photoresist layers. A 15:1 mixture of PDMS (Ellsworth) was spun on the pattern at 700 rpm, hard baked for 6 min at 80°C. The blank, sacrificial PDMS layer was then peeled and removed from the pattern.

A PCR program then ran for 60 cycles of 95°C for 30 s, and 72°C for 30 s. Standards for quantification were created by serial dilution of a 104-bp synthetic target equivalent to the bisulfite-converted locus.

Sample loading and digitization

The PCR and HRM master mixes were prepared to yield final working concentrations of 1.66 mM (NH₄)₂SO₄, 6.7 mM tris (pH 8.8), 2.7 mM MgCl₂, 1 mM β-mercaptoethanol, 300 nM primers (IDT; table S1), 200 μM dNTPs (Thermo Fisher Scientific), 1× ROX (Thermo Fisher Scientific), Platinum Taq DNA polymerase (0.04 U/μl) (Thermo Fisher Scientific), bovine serum albumin (New England BioLabs) (1 mg/ml), 0.01% Tween 20 (Sigma-Aldrich), and 1× EvaGreen (Biotium). This master mix was drawn into microcentrifuge tubing (Cole-Parmer) using a syringe. Because of the desiccation, a negative pressure differential existed inside the microfluidic chip seal. When the needle of the tubing punctured the seal, the sample was drawn into the wells. Partitioning fluid, consisting of 5 g of silicone oil and 1 g of PDMS (10:1), was then pressurized through the channels. Because of surface tension, the fluid did not enter the wells and served to isolate the reaction chambers and digitize the sample throughout the 4096 wells. During PCR, the partitioning fluid was pressurized at one end and sealed at the other. Furthermore, the PDMS in the oil solidified over the course of the PCR, producing a permanent barrier.

Digital PCR and digital melt

The chip was then placed on a flatbed thermal cycler (ProFlex). Thermal contact was ensured by use of FC-40 oil between the glass slide and the heating surface. A PCR program then ran for 60 cycles of 95°C, Tₐ (where Tₐ is the annealing temperature for the DREAMing primers), and 72°C for 30 s each. The annealing temperature, which controls the amplification bias of the primers to methylated versus unmethylated targets as previously described (21), was optimized for reduced bias to provide more uniform amplification of heterogeneously methylated epialleles (fig. S5). The Tₐ was 63°C for the p14ARF assay and 57°C for the NDRG4 assay. Next, the chip was removed from the heater, disconnected from the tubing, and taken to the digital melt platform (fig. S2). The chip was secured to a flatbed heater with tape. The wells were illuminated by an expanding blue LED array (Thorlabs). Wide field-of-view imaging captured the fluorescence of all 4096 wells simultaneously by the use of a Sony ILCE-7, a 50-mm lens (Canon), which was coupled to a green filter (Omega Optical). The chip underwent temperature ramping at a rate of 0.1°C/s from 70°C to 95°C. Images were captured at 1 Hz with a 0.8-s exposure. An unmethylated control was run before and after each serial dilution to verify the absence of contamination.

Image processing and data analysis

After collection, the images were aligned to correct for any thermally induced movement via an open-source automated program, AIR (33). The remainder of the analysis was performed with a custom-developed MATLAB script (fig. S4). The user selects the four corners of the chip to generate a virtual mask outlining the expected well locations. Once generated, the virtual mask was mapped to the acquired image via a homography transformation to correct for the nonparallel

Genomic and synthetic control DNA

All synthetic control DNA was obtained from Integrated DNA Technologies (IDT) and used at concentrations based on the manufacturer’s specifications. Human male genomic DNA (Promega) and EpiTect unmethylated control DNA (Qiagen) were used as unmethylated control genomic DNA. Enzymatically CpG-methylated HeLa genomic DNA (New England BioLabs) was used as a fully methylated control. Control genomic DNA was bisulfite-converted using the EZ DNA Methylation-Lightning Kit (Zymo Research) according to the manufacturer’s protocol and eluted into volumes ranging from 12 to 25 μl. Post-bisulfite treatment yields were quantified by MethLight using primer and probe sequences for β-actin recognizing both methylated and unmethylated templates: actin sense (5′-TAGGGATATATAGTTTGGGGAATTT-3′), actin antisense (5′-AAACACACATAAACACAAATTCAC-3′), spanning a 103-base pair (bp) region (chr7:5,532,169-5,532,271), and 100 nM probe (5′-A56-FAM\GTGGGGTTG\ZEN\GTGATGGAGGTTTAG\3IABkFQ\-3′). Assays were performed using 10× Master Mix to yield a final volume of 25 μl and final working concentrations of 16.6 mM (NH₄)₂SO₄, 67 mM tris (pH 8.8), 6.7 mM MgCl₂, 10 mM β-mercaptoethanol, 200 μM of each deoxynucleotide triphosphate (dNTP), and Platinum Taq polymerase (0.04 μU/μl) (Thermo Fisher Scientific). Cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s. Standards for quantification were created by serial dilution of a 104-bp synthetic target equivalent to the bisulfite-converted locus.
relationship between the signal and imaging surfaces. Finally, well-by-well transformations were performed by locally optimizing the signal over a 5 × 5 pixel neighborhood. The overall image contained 4240 × 2832 pixels, yielding approximately 200 pixels per well.

Once well locations were determined, the virtual mask was propagated throughout all of the collected images and captured the signal intensity of the 200 pixels in each well. Fluorescence intensity values within a well at each time point were checked for outliers and then averaged. Time points were then sorted by temperature, and each signal within 0.3°C was further averaged. Finally, a low-pass and Savitzky-Golay filter was performed on each well to produce a melt curve. The negative derivative of this signal was taken, and then corresponding peak(s) determined the melt temperature of the amplicon in each well. Only the right-most peak is used for discernment in each well to avoid any potential signal from heteroduplex formation. Simple thermal calibration was performed by taking advantage of the DREAMing assay principles (21), namely, a known background population of unmethylated targets, which had a consistent melt temperature and could thus be used for interexperimental alignment.

**Clinical samples**

All blood samples from patients diagnosed with mCRC were obtained through a study with approval by the MD Anderson Institutional Review Board, and all participants provided written informed consent (NCT01730586). Blood samples were collected at baseline from mCRC patients before clinical trial treatment. Blood samples of cancer-free individuals were obtained from outpatients of Johns Hopkins Community Physicians, with approval by the Johns Hopkins Medicine Institutional Review Board. All participants provided written informed consent. mCRC and cancer-free patient characteristics are provided in fig. S7B. All blood samples were collected in cell preparation tubes (BD) and processed within 1 hour with centrifugation (3000g) for 30 min, and then plasma was aliquoted to 1.8-ml cryog (3000g) and processed within 1 hour with centrifugation (3000g) for 30 min, and then plasma was aliquoted to 1.8-ml cryo-

All cfDNA was extracted using 2-ml NeoGeneStar Circulating Cell Free DNA kit (NeoGeneStar) according to the manufacturer’s instructions. Briefly, 2.0 ml of plasma was stabilized with pretreatment buffer and then digested in a solution containing 1× protease buffer and 100 μl of proteinase K (Invitrogen). DNA was then extracted with supplied chaotropic salts, washed by a series of magnetic decantation steps, and eluted into 20 μl of DNA elution buffer (Zymo Research). Extracted cfDNA was quantified by quantitative PCR using primers recognizing β-globin (forward primer: 5′-TGAAGGCTCATGCGAAGAAAG-3′; reverse primer: 5′-GAGGTGTGCTCAGGGTGACCA-3′). PCR performed using 10× PCR buffer (Thermo Fisher Scientific) to yield a final volume of 25 μl and a final working concentration of 3.5 mM MgCl₂, 200 μM of each dNTP, and Platinum Taq polymerase (0.04 U/μl) (Thermo Fisher Scientific). Cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 5 s, 65°C for 30 s, and 72°C for 30 s. Standards for quantification were created by serial dilution of human male genomic DNA (Promega). The resulting DNA was bisulfite-converted using the EZ DNA Methylation-Lightning Kit (Zymo Research) according to the manufacturer’s protocol and eluted into a final volume of 12 μl. Final bisulfite-treated–cfDNA yields were quantified by β-actin PCR, as described for control DNA above.

**MSP/MethyLight and ddPCR-MPS/MethyLight**

All MethyLight assays were performed using 10× Master Mix to yield a final volume of 25 μl and final working concentrations of 16.6 mM (NH₄)₂SO₄, 67 mM tris (pH 8.8), 6.7 mM MgCl₂ 10 mM β-mercaptoethanol, 200 μM of each dNTP, and Platinum Taq polymerase (0.04 U/μl) (Thermo Fisher Scientific). Each assay was validated by serial dilution to create a percent methylated reference standard curve ranging from 0.1 to 100% of bisulfite-converted methylated to unmethylated control genomic DNA (Promega). MethyLight assay validation results are provided in fig. S7.

The MSP/MethyLight assay (39) for NDRG4 was performed with 300 nM forward primer (5′-GGGTGAGGCGAAGGTATTTAC-3′), 300 nM reverse primer (5′-TAAATTATTACCGATTAACGCTGACCC-3′), spanning a 123-bp region (chr16:58,463,535-58,636,637), and 100 nM probe (5′-GGAGTTCGATTTAAGTGGTTGCTTGACGACGG-3′). Cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s using a CFX96 Touch Real-time PCR Detection System (Bio-Rad) and analyzed using the accompanying stock software CFX Manager.

The MethyLight and corresponding droplet digital assay for CDKN2A (p14ARF) was performed with 300 nM forward primer (5′-TCGGTTATTTTTTGTGGATGCC-3′), 300 nM reverse primer (5′-ACCCCTCCGGAATTCGAC-3′), spanning a 95-bp region (chr9:21,994,226-21,994,310), and 100 nM probe (5′-GGAGTTCGATTTAAGTGGTTGCTTGACGACGG-3′). MethyLight cycling conditions were 95°C for 5 min, followed by 50 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. MethyLight was performed using a CFX96 Touch Real-time PCR Detection System (Bio-Rad) and analyzed using the accompanying stock software CFX Manager.

For ddPCR, a master mix was prepared with Bio-Rad Supermix 1× according to Bio-Rad instructions. Mixed epiallelic targets were diluted to 40, 400, and 4000 copies per 20 μl, and background unmethylated targets were diluted to 2 million copies per 20 μl. ddPCR was performed in each dilution in duplicate, with and without the background DNA. Droplets were generated with the QX200 Droplet Generator (Bio-Rad), amplified in a 96-well plate (Bio-Rad) thermal cycler (Applied Biosystems), and detected via the QX200 Droplet Reader. Cycling conditions were 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 1 min, and 98°C for 10 min at a ramp rate of 2°C/s, as recommended by the manufacturer. Thresholding was performed across all reads by the proprietary Bio-Rad software to obtain the copy number detected per reaction volume (20 μl) (fig. S9).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/9/eaat6459/DC1

Table S1. Assay primers and synthetic targets.

Fig. S1. Ultrathin microfabrication.

Fig. S2. Digital melt platform.

Fig. S3. Illumination optimization.

Fig. S4. Pixel-space definition mapping.

Fig. S5. Annealing temperature on-chip optimization.

Fig. S6. NDRG4 copies detected in plasma samples.

Fig. S7. Clinical sample workflow, patient characteristics, and validation of MSP assay.

Fig. S8. Genomic validation of HYPER-Melt platform.

Fig. S9. Comparison of HYPER-Melt with ddPCR.

Movie S1. Loading and partitioning in the microfluidic device.

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