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Droplet-Based Microfluidic Systems for High-Throughput Single DNA Molecule Isothermal Amplification and Analysis

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We have developed a method for high-throughput isothermal amplification of single DNA molecules in a droplet-based microfluidic system. DNA amplification in droplets was analyzed using an intercalating fluorochrome, allowing fast and accurate "digital" quantification of the template DNA based on the Poisson distribution of DNA molecules in droplets. The clonal amplified DNA in each 2 pL droplet was further analyzed by measuring the enzymatic activity of the encoded proteins after fusion with a 15 pL droplet containing an in vitro translation system.

Digital PCR is based on the Poisson distribution of DNA molecules in microtiter plate wells (eq 1; in which $P(X = k)$ is the probability to have k DNA molecules per well, and λ is the mean number of DNA molecules per well).

$$P(X = k) = \frac{e^{-\lambda}}{k!}\lambda^k \quad (1)$$

At low λ (<0.3), the vast majority of wells contain no more than a single DNA molecule, and fitting the number of PCR competent wells to eq 1 allows the DNA concentration to be calculated. Digital PCR is used, for example, to detect low concentrations of mutations associated with colorectal cancer for diagnosis.[1] However, the large number of reactions required results in high reagent costs. Reaction volumes have been reduced by ~1000-fold (down to tens of nanolitres) using microfluidic systems by performing PCRs separated spatially in continuous flow[2,3] or within compartments defined by elastomeric valves.[4] However, single DNA molecules can also be compartmentalized in microdroplets in water-in-oil emulsions,[5] which act as microreactors with volumes down to 1 fL.[6] Digital PCR in emulsions (emulsion PCR) is already used to quantify rare mutations using BEAMing[7] and to prepare the template for two commercialized "next-generation" DNA sequencing systems.[8]

Direct, quantitative screening using emulsion PCR is, however, compromised by the polydispersity of bulk emulsions. Furthermore, it is difficult to add reagents to droplets after they are formed,[6] which limits the range of assays that can be performed on the amplified DNA. However, both of these problems can potentially be overcome using droplet-based microfluidic systems that allow the production of highly monodisperse droplets[9] and pairwise droplet electrocoalescence.[10−14]

Digital PCR has previously been used to quantify DNA in droplets in microfluidic systems.[15−18] This manuscript, however, describes the digital quantification of DNA using a droplet-based microfluidic system and isothermal "hyperbranched rolling circle

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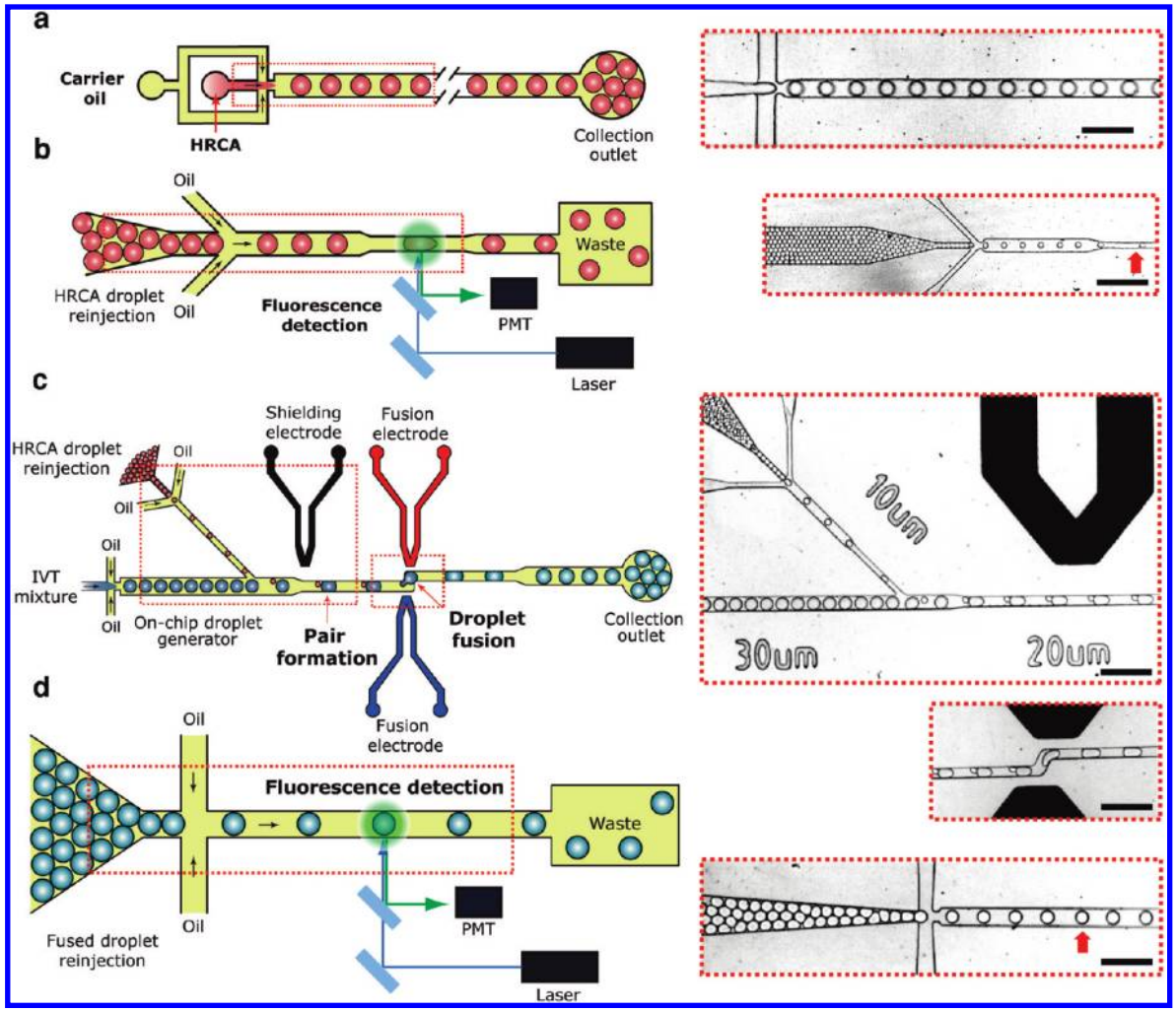

Figure 1. The microfluidic devices. (a) HRCA droplet generation device. An HRCA system containing the plasmid DNA was emulsified using HFE-7500 fluorinated oil with 2% (w/w) EA surfactant. (b) HRCA droplet analysis device. After incubation off-chip, HRCA droplets were reloaded into the device and spaced with HFE-7500 fluorinated oil, and their fluorescence was monitored using PMTs. (c) Droplet fusion device. HRCA droplets were reloaded, spaced by HFE-7500 fluorinated oil containing 2% (w/w) EA surfactant, and paired with IVT droplets generated on-chip. Droplet pairs were electrocoalesced between two electrodes (red and blue) by application of an AC field (30 kHz, 600 V) while shielding electrodes (black) prevented unwanted electrocoalescence. (d) Fused droplet analysis device. Fused droplets were reinjected and spaced with HFE-7500 fluorinated oil, and their fluorescence was monitored. Devices were fabricated in PDMS using soft lithography. The scale bar is 100 μm, and red arrows show the detection points. The corresponding movies can be found as Supporting Information.

amplification" (HRCA).[19] In addition, the clonal amplified DNA in each droplet was further analyzed by measuring the activity of the encoded enzymes after fusion with a droplet containing an in vitro translation (IVT) system.

EXPERIMENTAL SECTION

Microfluidic Devices. Microfluidic devices were fabricated by patterning 10-μm-deep channels for the HRCA droplets generator device (Figures 1a and S-1 (Supporting Information)), 15-μm-deep channels for HRCA analysis device (Figures 1b and S-2 (Supporting Information)), or 20-μm-deep channels for the fusion and IVT analysis devices (Figure c and d and Figures S-3 and S-4 (Supporting Information)) into poly(dimethylsiloxane) (PDMS) using soft lithography.[20] Briefly, a mold of SU-8 resist (MicroChem Corp., Newton, MA) was fabricated on a silicon wafer (Siltronix, Archamp, France) by UV exposure (MJB3 contact mask aligner; SUSS MicroTec, Garching, Germany) through a photolithography mask (Selba SA, Versoix, Switzerland) and subsequent development (SU-8 developer; MicroChem Corp.). Curing agent was added to PDMS base (Sylgard 184 silicone elastomer kit; Dow Corning Corp., Lyon, France) to a final concentration of 10% (v/v), mixed, and poured over the mold to a depth of 5 mm. Following cross-linking at 65 °C for ~12 h, the PDMS was peeled off the mold, and the input and output ports were punched with a 0.75-mm-diameter Harris Uni-Core biopsy punch (Electron Microscopy Sciences, Hatfield, PA). Particles of PDMS were cleared from the ports using pressurized nitrogen gas. The structured side of the PDMS slab was bonded to a 76 × 26 × 1 mm glass microscope slide (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) by exposing both parts to an oxygen plasma (PlasmaPrep 2 plasma oven; GaLa Instrumente GmbH, Bad Schwalbach, Germany) and pressing them together. For the droplet

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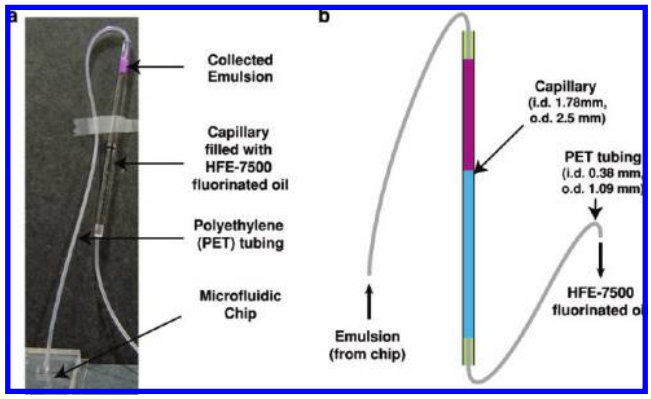

Figure 2. Capillary-based emulsion collecting system. (a) Photograph of the capillary-based system connected to a microfluidic chip. A glass capillary is interfaced with the chip by polyethylene tubing. (b) Schematic representation of the capillary-based collecting system. The capillary is represented in the collecting configuration with the syringe disconnected. The emulsion (purple) coming from the chip enters into the capillary filled with HFE-7500 fluorinated oil (blue) and pushes it out. PDMS adapters are shown in green, and polyethylene (PET) tubing, in gray.

fusion device, indium tin oxide (ITO)-coated glass slides were used (Delta Technologies, Stillwater, MN). Finally, the devices were coated with a commercial hydrophobic surface coating agent (Aquapel, PPG Industries, Pittsburgh, PA) and subsequently flushed with N_2. Electrodes used for fusion were fabricated in close proximity to the microfluidic channels, as described previously.[21] The patterned electrodes were filled with metal. The device was heated to 85 °C and $^{51}In/^{32.5}Bi/^{16.5}Sn$ low-temperature solder (Indium Corporation, Singapore) was melted inside the electrode channels. Electrical connections with the solder electrodes were made with short pieces of electrical wire (Radiospares, Beauvais, France). Dimensions of the microchannels are described in Figures S-1−4 (Supporting Information).

Liquids were pumped into microfluidic devices using standard-pressure infuse/withdraw PHD 22/2000 syringe pumps (Harvard Apparatus Inc., Holliston, MA). Syringes were connected to the microfluidic device using 0.6 × 25 mm Neolus needles (Terumo Corporation, La Chaussée St. Victor, France) and PTFE tubing with an internal diameter of 0.56 mm and an external diameter of 1.07 mm (Fisher Bioblock Scientific, Illkirch, France).

Fabrication of Capillary-Based Emulsion Collecting Systems. The capillary-based emulsion collecting system is shown in Figure 2. A disposable 200 μL glass capillary of internal diameter of 1.78 mm and an external diameter of 2.5 mm (Duran, Fisher Scientific, Illkirch, France) was assembled with PDMS connectors cast at both ends with 0.8-mm-diameter holes to permit the connection of thin (internal diameter of 0.38 mm and an external diameter of 1.09 mm) polyethylene (PET) tubing PE-20 (Intramedic, Becton Dickinson, Franklin Lakes, NJ). The capillary was connected to a syringe containing HFE-7500 fluorinated oil (3-ethoxy-1,1,1,2,3,4,4,5,5,6,6,6-dodecafluoro-2-(trifluoromethyl) hexane; 3M, St. Paul, MN) using a length of PET tubing. A second, shorter (10 cm) length of PET tubing was connected to the other end of the capillary, and the whole assembly was primed with HFE-7500 fluorinated oil from the syringe.

DNA Preparation. The plasmid pIVEX2.2EM-*lacZ*[22] was replicated in *Escherichia coli* XL-1 blue cells (Stratagene, La Jolla, CA), purified using a Midiprep kit (Qiagen, Germantown, MD), and its concentration was estimated both on an agarose gel and by measuring the absorbance at 260 nm using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). DNA dilutions were performed in 20 ng/μL of carrier tRNA (Ambion, Foster City, CA).

Rolling Circle Amplification. HRCA amplifications were performed using the commercial "Illustra GenomiPhi V2 DNA Amplification Kit" (GE Healthcare, Saclay, France). Bulk amplifications were performed according to the manufacturer's instruction for 4 h at 30 °C.

When HRCA reactions were performed in emulsions, the amplification mixture was further supplemented with 1 μg/μL of purified BSA (New England Biolabs, Ipswich, MA), 2.3 μg/mL of ds-DNA intercalating dye (RDT-D1, RainDance Technologies, Lexington, MA), and 1 mg/μL 70 000 kDa dextran-Texas Red conjugate (Invitrogen, San Diego, CA). The aqueous phase was loaded into PTFE tubing (0.56 × 1.07 mm internal/external diameter, Fisher Bioblock Scientific) kept on ice throughout the emulsification process, and the tubing was connected to a syringe filled with 98% decane (Fluka, Sigma-Aldrich, Lyon, France). The HRCA mixture was injected into the droplet generator microfluidic device (Figures 1a and S-1 (Supporting Information)) using a syringe pump (PHD 2000, Harvard Apparatus, Holliston, MA) at a flow rate of 100 μL/h. Droplets were generated by flow focusing of the resulting stream with HFE-7500 fluorinated oil containing 2% (w/w) EA surfactant (RainDance Technologies, Lexington, MA), which is a PEG−PFPE amphiphilic block copolymer,[23] at a flow rate of 250 μL/h. The rate of droplet production was ~14 kHz. The droplet volume was calculated by dividing the flow rate of the aqueous phase by the droplet production frequency (determined by using a Phantom V4.2 high-speed camera).

When the HRCA reactions were analyzed by agarose gel electrophoresis, the emulsions were collected in a microtube under mineral oil to prevent evaporation. After incubation, the mineral oil was drained out, and 1/10 volume of Emulsion Destabilizer (RainDance Technologies, Lexington, MA) was added to 1 volume of emulsion. After a quick vortexing and centrifugation, the aqueous phase was transferred to a new tube, and the Phi29 polymerase was inactivated by heating the mixture for 20 min at 65 °C. HRCA products were then monomerized by incubating 20 μL of HRCA mixture with 10 U of *Eco*RI (Fermentas, Gmbh, St Leon-Rot, Germany) overnight at 37 °C. Digested products were analyzed on a 1% agarose gel stained with ethidium bromide.

When the HRCA reactions were analyzed by monitoring droplet fluorescence, the emulsions were collected in capillaries filled with HFE-7500 fluorinated oil and incubated 4 h at 30 °C. Droplet fluorescence analyses were carried out by reinjecting HRCA emulsions from capillaries into the HRCA droplet analysis

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device (Figures 1b and S-2 (Supporting Information)) at a flow rate of 10 μL/h. To allow data acquisition on isolated droplets, the droplets were spaced with surfactant-free HFE-7500 fluorinated oil at a flow rate of 150 μL/h, and droplet fluorescence was monitored using the optical setup shown in Figure S-5 (Supporting Information) by focusing the 488 nm laser beam in the middle of a 10-μm-wide and 15-μm-deep channel of the device. Fluorescence was measured using PMTs, and the signal output from the PMTs was analyzed using a PCI-7831R Multifunction Intelligent DAQ card (National Instruments Corporation, Austin, TX) executing a program written in LabView 8.2 (FPGA module, National Instruments Corporation). The data acquisition rate for the system was 100 kHz.

Enzymatic Assay of the in Vitro Translated *lacZ* in Droplets. The *lacZ* gene carried by the purified pIVEX2.2EM-*lacZ* plasmid,[22] or the amplified plasmid was expressed, respectively, by directly mixing plasmid DNA with the IVT system or by fusing HRCA product-containing droplets with droplets containing the IVT system. The IVT system contained 0.7 volume of *E. coli* extract (EcoProT7 kit, Novagen, Merck, Darmstadt, Germany), 300 μM of methionine, 100 μM fluorescein di-β-D-galactopyranoside (Euromedex, Souffelweyersheim, France), 1 μM fluorescein, and 0.1 mg/μL 70 000 kDa dextran-Texas Red conjugate (Invitrogen, San Diego, CA). The mixture was loaded in PTFE tubing kept on ice throughout the emulsification process, and the tubing was connected to a syringe filled with 98% decane (Fluka, Sigma-Aldrich, Lyon, France).

After *lacZ* gene HRCA amplification (4 h at 30 °C), the emulsion was reinjected from the capillary into a droplet fusion device (Figures 1c and S-3 (Supporting Information)) at 20 μL/h and spaced with HFE-7500 fluorinated oil containing 2% w/w EA surfactant at 120 μL/h (~1.7 kHz). On-chip droplets (15 pL volume) were generated on the droplet generation module at ~1.85 kHz using the flow rates at 100 μL/h for IVT mixture and 100 μL/h for HFE-7500 fluorinated oil containing 2% (w/v) EA-surfactant. To obtain the highest frequency (~95%) of one-to-one fusion events, the flow rate of HFE-7500 fluorinated oil used to space reinjected HRCA droplets was adjusted to match the frequencies of reinjected droplets and droplets produced on-chip. Electrocoalescence of droplets[10−14] was performed for 30 min at a rate of ~2000 fusion events/s by applying an electric field of 600 V AC at 30 kHz across electrodes using a Model 623b high-voltage amplifier (Trek, Inc.; BF: OPTiLAS SAS, Evry, France). After droplet electrocoalescence, over 10^6 fused droplets were collected into a single glass capillary connected to the chip. The glass capillary was then transferred to a 37 °C air thermostat and incubated for 2 h to allow transcription and translation to occur in the droplets. Fused droplets were then reloaded into a fused droplet detection device (Figures 1d and S-4 (Supporting Information)) at 40 μL/h and spaced by HFE-7500 fluorinated oil (without surfactant) at 120 μL/h, resulting in a reinjection frequency of ~0.9 kHz. The 488 and 532 nm laser beams (Figure S-5 (Supporting Information)) were focused in the middle of the 40-μm-wide and 20-μm-deep reinjection channel (~250 μm beyond the flow focusing junction). This allowed the measurement of both β-galactosidase activity (green) and Texas Red (orange) in the droplets. Fluorescence-emitted light from ~10^5 droplets was recorded using two photomultiplier tubes (PMTs).

Confocal Microscopy Imaging. Droplets were placed between two 0.17-mm-thick microscope coverslips, and images were acquired with a Zeiss (Jena, Germany) LSM510 laser-scanning confocal microscope equipped with C-Apochromat 20× (n.a. 0.8) or 40× (n.a.1.1) water immersion objectives in multitrack mode. RDT-D1 was excited at 488 nm using an argon laser, and emission spectra were recorded from 505 to 550 nm. Texas Red fluorescence was excited at 543 nm using a helium−neon 543 nm laser, and emission spectra were recorded at wavelengths higher than 585 nm. Images were processed with the Zeiss LSM Image Browser, version 2.50.0929, software.

Estimation of Ribosome Content of the IVT System. Total RNA was extracted by mixing 1 volume of IVT mixture (35 μL of bacterial extract and 15 μL of water) with 1 volume of phenol/chloroform/isoamylic alcohol (25/24/1). Different dilutions of the recovered aqueous phase were run on an 8% denaturating acrylamid gel, together with 7−500 ng of a standard RNA of the same size. The intensity of the 120-bp band (5S RNA) was measured (Gel Logic 200 Imaging System, Kodak; Carestream Health Corp., New York, NY) and compared to those of the standard.

Measures to Minimize Contamination. To minimize contamination by exogenous DNA, the components of the HRCA kit were aliquoted upon arrival and stored at −80 °C. A new aliquot was used for each set of experiments. Dilutions were performed in DNase-, RNase-free, DEPC-treated water (MP Biomedicals, Illkirch, France), and all DNA manipulations prior to amplification were performed in a UV-treated environment (Biocap DNA/RNA hood, Fisher Bioblock Scientific). A new device was used for each set of experiments, going from the more diluted to the more concentrated DNA sample to limit carry-over effects. In addition, all the experiments were carried out using certified DNA-free, sterilized, filtered pipet tips.

RESULTS AND DISCUSSION

In this study, we used a microfluidic system composed of separate devices (chips) (Figure 1) for droplet generation, fusion, and detection, allowing isothermal amplification and analysis of the 6 kb pIVEX2.2EM-*lacZ* plasmid[22] encoding β-galactosidase.

A Hyperbranched Rolling Circle Amplification (HRCA) system (Illustra GenomiPhi V2; G.E Healthcare, Saclay, France) was first tested in bulk assays using as template the pIVEX2.2EM-*lacZ* plasmid at concentrations ranging from 35 to 0.07 pg/μL. After incubation and polymerase inactivation, the amplification products were monomerized by *Eco*RI digestion and analyzed by agarose gel electrophoresis (Figure 3). Efficient amplification was observed at DNA concentrations down to 0.07 pg/μL, confirming the high sensitivity of HRCA.[24] Because the HRCA system uses random hexamer primers, it can potentially amplify any DNA in a nonspecific manner. Consequently, we followed the procedure described in the Experimental Section to minimize the contamination by exogenous DNA molecules. For applications in which avoiding contamination is of paramount importance (e.g., diagnosis

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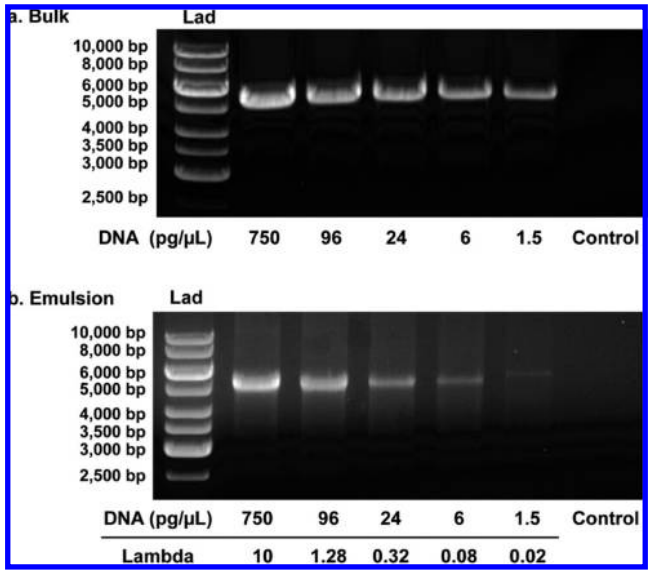

Figure 3. Gel analysis of bulk and emulsified HRCA reactions. Amplifications of the plasmid pIVEX 2.2EM-*lacZ* were performed in bulk (a), or in droplets (b). After incubation, the polymerase was inactivated, and the amplification products monomerized and analyzed on 1% agarose gel stained with ethidium bromide. The GeneRuler 1kb DNA Ladder (Fermentas) was used as standard (Lad), and the size of each band is given. The DNA concentration used to initiate the amplification is indicated under each picture, and theoretical λ values are given for emulsified reactions. Control lanes correspond to reactions performed with a DNA concentration corresponding to $\lambda = 10$ but for which Phi29 polymerase was omitted.

or library screening), a more thorough approach, similar to the one recently described for single-cell DNA sequencing methods,[25] could be used.

The same set of experiments was then carried out in an emulsion. HRCA mixtures supplemented with 1% BSA were dispersed in HFE-7500 fluorinated oil containing 2% (w/w) EA surfactant using a microfluidic flow-focusing device with a 10 μm nozzle (Figures 1a and S-1 (Supporting Information)) to produce a highly monodisperse emulsion of 2 pL droplets at 14 kHz. The emulsion was collected in a microtube under mineral oil to prevent evaporation and incubated 4 h at 30 °C. The emulsion was then broken with Emulsion Destabilizer (RainDance Technologies), the aqueous phase was recovered, the polymerase was inactivated, and the HRCA products were monomerized. Gel analysis revealed that the yield of amplified DNA was almost identical in droplets and in bulk at DNA concentrations corresponding to an average starting number of plasmid molecules per droplet (λ) >1 (Figure 3). The yield of DNA is limited by the available dNTPs, primers, or both, in both cases. However, at $\lambda < 1$, as expected, the yield of amplified DNA in droplets decreases rapidly in droplets compared to in bulk due to the presence of droplets that contained no plasmid DNA, which reduces the quantity of available dNTPs and primers. The yield of amplified DNA from emulsions in which all ($\lambda = 10$) or the majority ($\lambda = 1.28$) of droplets were initially occupied was quantified by agarose gel electrophoresis. We estimated that at both $\lambda = 10$ and $\lambda = 1.28$, each droplet contained ~30 000 HRCA monomerized products, indicating that a single encapsulated plasmid was amplified by ~30 000-fold.

The content of each droplet was then individually monitored by further supplementing the HRCA system with a ds-DNA intercalating dye (RDT-D1, RainDance Technologies), which becomes highly green fluorescent when intercalated into double-stranded DNA, to identify droplets where amplification occurred and with a dextran−Texas Red conjugate as an internal standard. A 2-step strategy was used (Figure 4a): (i) As above, the HRCA mixtures were compartmentalized in 2 pL droplets, but were collected this time in a glass capillary (Figure 2) and placed for 4 h at 30 °C. Droplet order is not strictly maintained in the capillary; however, the creaming and packing of droplets means that there is a tendency for the last droplets in to be the first out. However, droplet production and reinjection was fast enough (less than 10 min for 10^7 droplets) to make the difference in incubation times between the droplets insignificant. For applications in which larger numbers of droplets have to be collected, the capillary can be connected to a Peltier-based temperature control device (CP-031, TE-technology, Traverse City, MI), allowing the synchronization of reactions in droplets by collecting them at 4 °C and activating the reactions by switching the temperature to 30 °C (for HRCA) or 37 °C (for IVT). (ii) After incubation, the capillary was connected to the analysis chip (Figures 1b and S-2 (Supporting Information)), the droplets were reinjected at 4−8 kHz and spaced using HFE-7500 fluorinated oil, and the fluorescence intensity of the droplets was monitored using an optical setup comprising a 488 nm laser and PMTs measuring epifluorescence at 525 nm (green) and 585 nm (orange) to detect amplified DNA and dextran−Texas Red, respectively. As expected, this analysis revealed single major peaks, corresponding to either all-green fluorescent or all-nonfluorescent droplets with 35 pg/μL DNA ($\lambda = 10$) or the control experiment in which the polymerase was omitted (Figure 4b and e), respectively. When using DNA concentrations from 0.07 to 4.5 pg/μL ($\lambda = 0.02-1.28$), two discrete populations corresponding to nonfluorescent (~25 RFUs) and green-fluorescent droplets (200−320 RFUs) were distinguishable (Figures 4c, d and S-6 (Supporting Information)). The small population between the peaks may result from abortive amplification of damaged plasmids. Consequently, only droplets in the positive peak were used to determine droplet occupancies. These occupancies were further confirmed by confocal microscopy (Figures 4 and S-6 (Supporting Information)). The mean fluorescence intensity of positive droplets decreased as DNA concentration and droplet occupancy increased (Figure 4f) due to passive transfer of RDT-D1 between the droplets and its uptake by the amplified DNA in occupied droplets. The occupancy was a Poisson distribution controlled by DNA concentration, and the experimental λ values closely fitted the theoretical ones calculated from the template DNA concentration ($R^2 = 0.903$) (Figure 4g). Hence, the technique allows accurate "digital" quantification of the template DNA.

The amplified DNA was then further characterized by expressing the *lacZ* gene carried on the plasmid. We first tested

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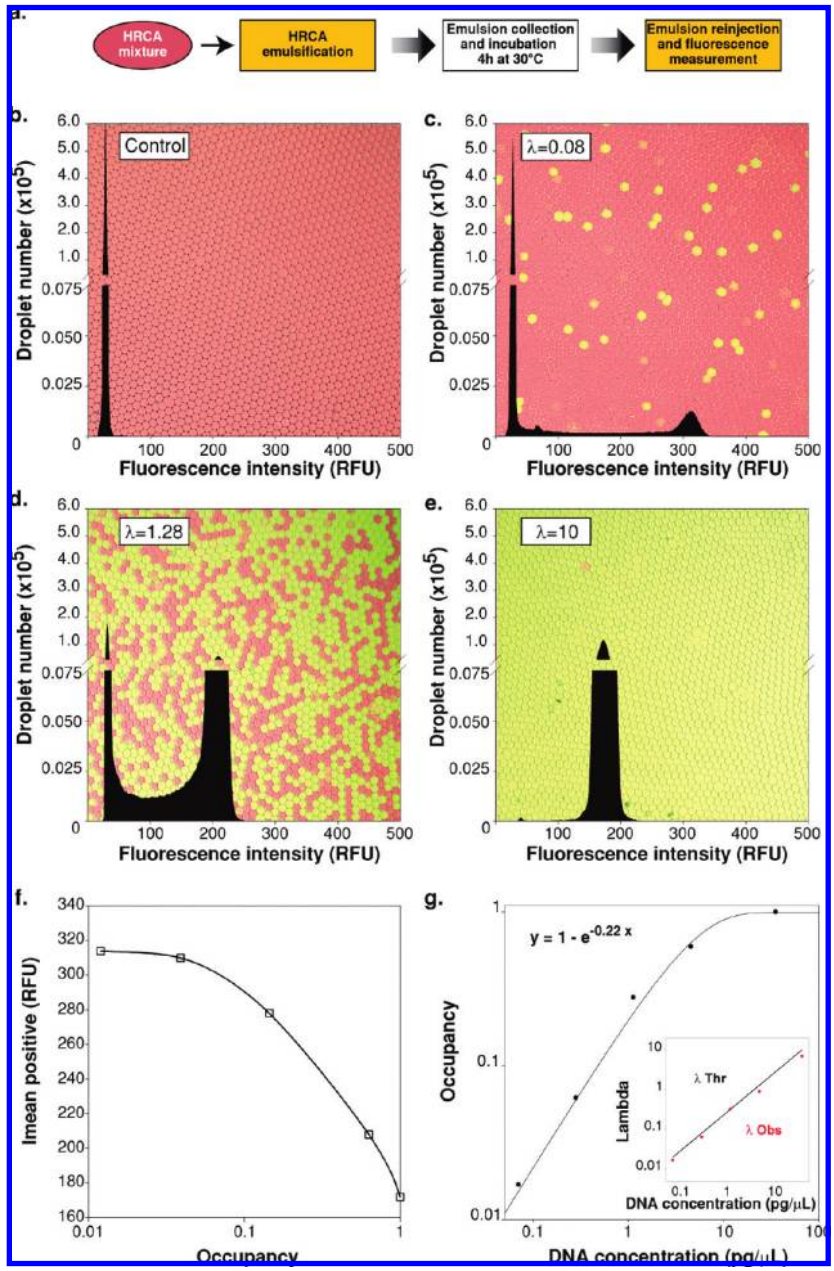

Figure 4. Characterization of digital HRCA reactions. (a) Overview of operations. (b−e) Histograms of green fluorescence, normalized by the orange fluorescence of the internal standard (dextran−Texas Red), of 10^6 droplets. Theoretical λ values are indicated, and confocal images of emulsions are shown (backgrounds). The control reaction was performed at $\lambda = 10$ but without polymerase. (f) The mean intensities (Imean) of the positive droplets versus droplet occupancies. (g) A curve described by the Poisson equation (occupancy $= 1 - e^{-\lambda}$) was fitted to experimental data using SigmaPlot (Systat Software GmgH, Erkrath, Germany). Inset: λ values derived from experimental data (red dots) and theoretical λ values (black line) plotted versus DNA concentration.

the activity of compartmentalized IVT reactions by coencapsulating purified plasmid DNA with a coupled in vitro transcription/translation (IVT) system (EcoPro T7, Novagen), a fluorogenic β-galactosidase substrate (fluorescein-di-β-D-galactopyranoside; FDG) and dextran−Texas Red conjugate. A DNA concentration corresponding to that of an amplified DNA (i.e., $\lambda = 20\,000$) was used to produce 1.3 million 15 pL droplets (20 μL of aqueous phase) that were collected on ice and transferred to a microtiter plate. The production of fluorescein (due to β-galactosidase activity) was monitored for 2 h at 37 °C in a spectrophotometer. An equivalent volume (20 μL) of bulk reaction was monitored in parallel and, as shown in Figure 5a, the rate of appearance of β-galactosidase activity was identical for both bulk and emulsified IVT at $\lambda = 20\,000$, demonstrating that IVT was not affected by the confinement in droplets. Interestingly, the same experiments performed with a DNA concentration equivalent to that prior to amplification (i.e., $\lambda = 2$, where 87% of the droplets contained at least one DNA molecule) gave no detectable β-galactosidase activity (in bulk or in emulsion). This observation was further confirmed by an experiment in which the fluorescence of each droplet was measured (Figure 5b) by reinjecting droplets into the device

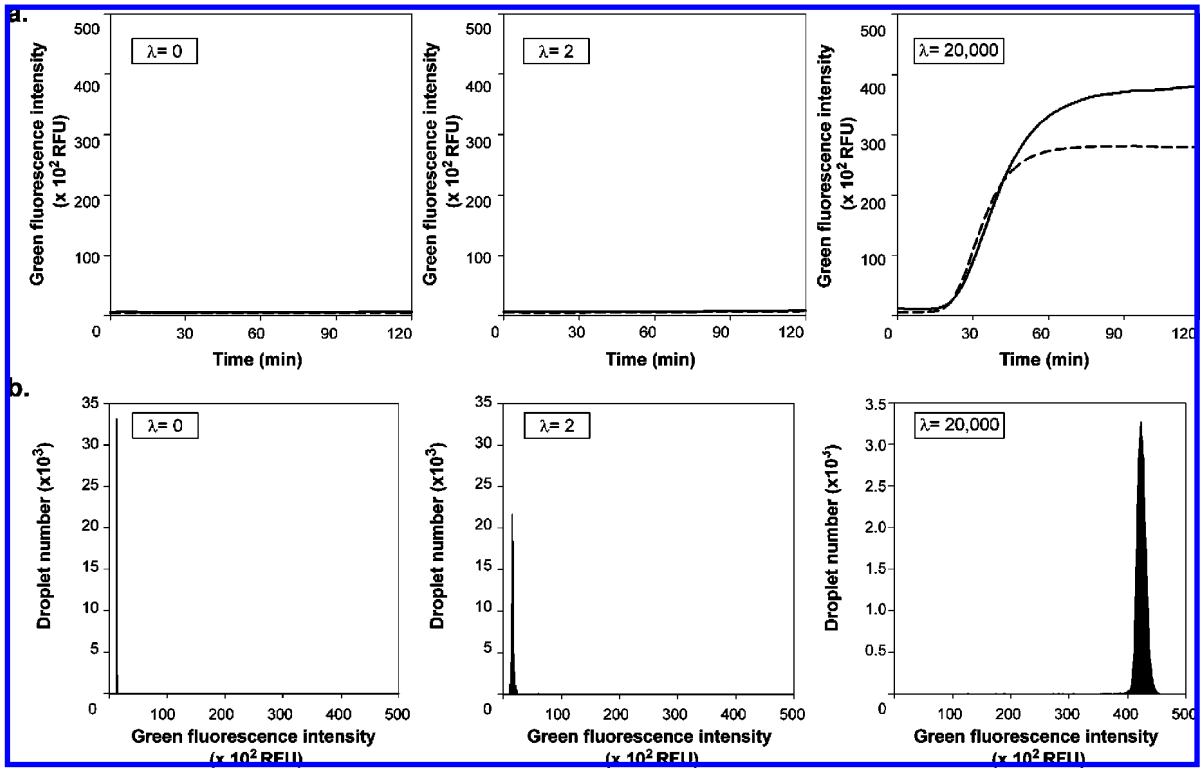

Figure 5. In vitro transcription/translation of *lacZ* genes carried by the plasmid pIVEX2.2EM-*lacZ* in droplets. (a) Kinetic monitoring of in vitro transcription/translation of *lacZ* genes. IVT systems containing FDG, dextran−Texas Red conjugate, and different plasmid concentrations corresponding to λ values of 0, 2, or 20 000 were either encapsulated in 20 pL droplets (solid lines) or kept in a bulk format (dashed lines), and the green fluorescence was monitored for 2 h at 37 °C in microtiter plate wells. (b) Different plasmid concentrations corresponding to λ values of 0, 2, or 20 000 were encapsulated in 20 pL droplets containing the IVT system, FDG, and dextran-Texas Red conjugate. After 2 h at 37 °C, the droplets were reinjected into the microfluidics device (Figures 1d and S-4 (Supporting Information)), and the green fluorescence intensity was measured.

shown in Figures 1d and S-4 (Supporting Information). Altogether, these results demonstrate that a successful amplification (λ = 20 000) is necessary to observe β-galactosidase activity whereas without amplification (λ = 2), a single *lacZ* gene does not lead to efficient production of β-galactosidase. We calculated, on the basis of catalytic activity, that the yield of β-galactosidase reached a plateau at a DNA concentration equivalent to λ = 2500, corresponding to ~1700 molecules of β-galactosidase per gene (Supporting Information, and Figure S-5). This yield is close to that expected from the EcoPro T7 IVT system (~300 proteins/gene [28 μg of β-galactosidase produced from 2 μg of gene] quoted by the supplier[26]). However, at lower DNA concentrations, the yield of active β-galactosidase dropped more rapidly than the decrease in DNA concentration (a 2800-fold drop in activity from λ = 500 to λ = 5), most likely due to the inefficient assembly of tetrameric β-galactosidase at low subunit concentrations. A yield of 1700 β-galactosidase molecules per gene is low compared to 30 000 green fluorescent protein (GFP) molecules expressed from single genes in droplets reported recently.[27] However, this difference can most likely be attributed to the fact that β-galactosidase is a large tetrameric protein of (4 × 116 kDa), whereas GFP is a 27 kDa monomeric protein, and hence, the yield of protein per gene by mass is almost identical for β-galactosidase and GFP (mass β-galactosidase per gene/mass GFP per gene = 0.97). The low yield of active β-galactosidase at low λ led us to perform fusion experiments between HRCA droplets with droplets containing an IVT system.

We used plasmid DNA at λ = 0.25 to minimize encapsulation of more than 1 plasmid/droplet ($P_{(X>1)}$ = 0.026). An emulsion in which the polymerase was omitted was used as the control. The procedure involved three steps (Figure 6a): (i) HRCA systems supplemented with 1% BSA and dextran−Texas Red were encapsulated in 2 pL droplets at 14 kHz using the device shown in Figures 1a and S-1 (Supporting Information), collected in glass capillaries (Figure 2), and incubated 4 h at 30 °C. (ii) These droplets were reinjected into the droplet fusion device at ~1.7 kHz (Figures 1c and S-3 (Supporting Information)). In parallel, the IVT system supplemented with FDG was encapsulated in 15 pL droplets on the same device (~1.85 kHz). Pairs of HRCA and IVT droplets were fused by electrocoalescence,[10−14] collected in a glass capillary, and incubated 2 h at 37 °C. Model experiments using fluorescent dyes demonstrated that the device was highly efficient (see the Supporting Information, Figure S-7): with an 11% excess of on-chip droplets, 95% of the reinjected droplets were fused one-to-one with a droplet generated on-chip (Figure 6b). (iii) Finally, fused droplets were reinjected into the fused droplet detection device, and the fluorescence intensity of each droplet was monitored using the device shown in Figures 1d and S-4 (Supporting Information).

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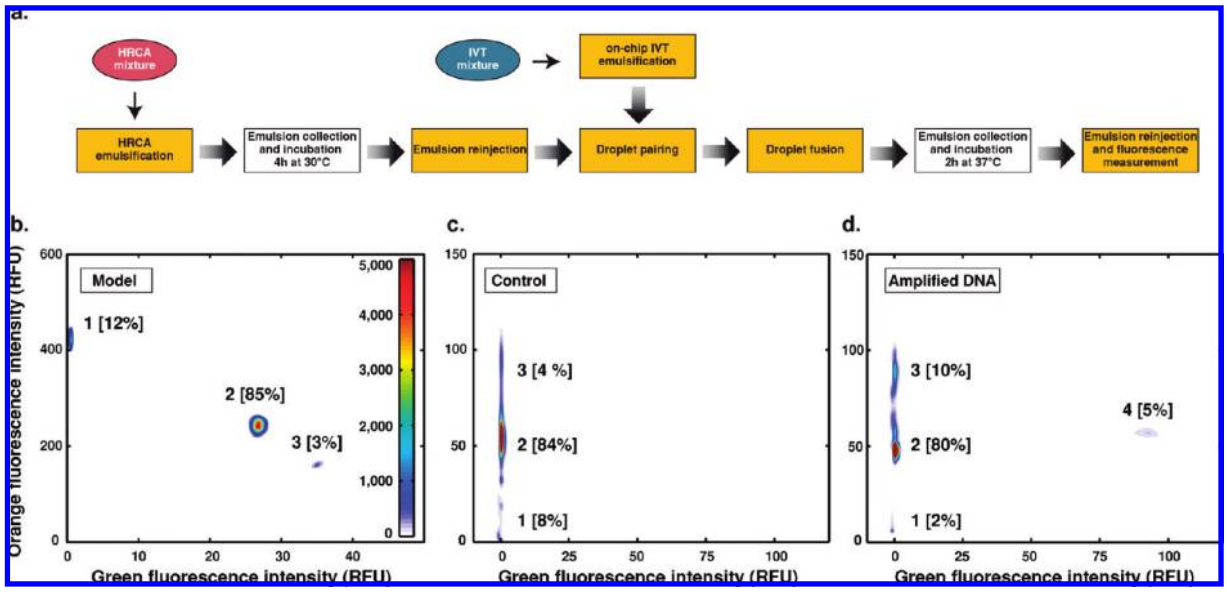

Figure 6. β-Galactosidase expression in fused droplets. (a) Overview of operations. (b) Model experiment. Reinjected droplets (2 pL) containing 10 μM fluorescein (green fluorescence) and 50 mM methylene blue were fused with 10 pL droplets generated on-chip containing 100 μM resorufin (orange fluorescence). The lower orange fluorescence in fused droplets was due to absorbance by the methylene blue. (c−d) HRCA reactions, containing Texas Red (orange fluorescence), were performed in 2 pL droplets at $\lambda = 0.25$ in the absence (c) or presence (d) of DNA polymerase. After *lacZ* gene amplification, these droplets were fused with 15 pL droplets containing an IVT system and FDG (which is transformed into fluorescein (green fluorescent) by β-galactosidase) and incubated, and the fluorescence of 150 000 droplets was analyzed. Unfused IVT droplets (population 1) and droplets fused one-HRCA-to-one-IVT (populations 2 and 4) and two-HRCA-to-one-IVT (population 3) are identified, and the percentage of the total population is indicated.

The orange fluorescence due to Texas Red in the HRCA droplets allowed pairwise-fused IVT droplets (1 HRCA/1 IVT) to be distinguished from nonfused and double-fused (2 HRCA/1 IVT) droplets. The green fluorescence resulting from FDG hydrolysis allowed droplets containing in vitro translated β-galactosidase to be identified. Fluorescein (1 μM) was added to the IVT, allowing for the detection of nonfused IVT droplets (population 1 in Figure 6c and d). Without prior plasmid DNA amplification (Figure 6c) or when plasmids were directly emulsified in IVT mixture at $\lambda = 2$, no activity was observed (see above). The similar behavior of reactions in bulk and in emulsion ruled out an inhibitory effect of compartmentalization on IVT. However, when plasmid DNA was amplified, a green-fluorescent droplet population appeared (Figure 6d), indicating the presence of active β-galactosidase. Hence, single molecules of DNA compartmentalized in droplets can be amplified and the enzymatic activity of the encoded protein measured.

Six percent of pairwise-fused droplets (85% of the total droplets) contained active β-galactosidase, rather than 22%, as expected from the direct measurement of DNA amplification in droplets (Figure 4g). This effect cannot be due to stochastic variations in the number of ribosomes in droplets, since gel quantification of 5S rRNA content of the IVT system indicated that each droplet contains, on average, 10^6 ribosomes (data not shown). The origin of this effect is still under investigation.

CONCLUSIONS

This is the first time that such a high-throughput, on-chip analysis (up to 8 kHz) was achieved for clonal DNA amplification using microsystems. The use of an isothermal amplification system (HRCA) avoids many complications linked to the thermocycling of PCRs. Furthermore, the ability not simply to measure DNA amplification but also to measure the activity of the encoded enzymes after IVT greatly increases the range of potential applications. IVT is widely used for the rapid identification of gene products (proteomics), localization of mutations causing truncated gene products, protein folding studies, and incorporation of modified amino acids.[28] Many of these applications could potentially be implemented in droplet-based microfluidic systems, with massive reagent cost savings: 10^6 IVT reactions in 20 pL droplets costs only $4.50, as compared to $1 137 500 for 10^6 5 μL IVT reactions in microtiter plates (see Supporting Data).

In the future, by integrating further microfluidic modules, even greater functionality could be achieved. For example, droplets can be sorted or triggered on fluorescence using dielectrophoresis,[29,30] which would allow the development of a completely in vitro system for directed evolution. The preamplification of genes before translation is likely to be important for such applications because, although the translation of single GFP genes in droplets in microfluidic systems has been reported,[27] we were unable to detect the expression of single *lacZ* genes, despite the fact that β-galactosidase is a highly active enzyme ($k_{cat} = 187\ s^{-1}$, $K_M = 150\ \mu M$) which is efficiently translated in vitro.[31−33]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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