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TITLE:

Particle templated emulsification enables microfluidic-free droplet assays.

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KEYWORDS:

digital droplet PCR, single molecule encapsulation, particle templated emulsification, droplet microfluidics, single cell analysis, digital biology, hydrogel particles

SUMMARY:

Water-in-oil droplet assays are useful for analytical chemistry, enzyme evolution, and single cell analysis, but typically require microfluidics to form the droplets. Here, we describe particle templated emulsification, a microfluidic-free approach to perform droplet assays.

ABSTRACT:

Reactions performed in monodispersed droplets afford enhanced accuracy and sensitivity compared to equivalent ones performed in bulk. However, the requirement of microfluidics to form controlled droplets imposes a barrier to non-experts, limiting their use. Here, we describe particle templated emulsification, an approach to generate monodisperse droplets without microfluidics. Using templating hydrogel spheres, we encapsulate samples in monodispersed droplets by simple vortexing. We demonstrate the approach by using it to perform microfluidic-free digital PCR.

INTRODUCTION:

Droplet microfluidics leverages compartmentalization in picoliter droplets to increase the sensitivity and accuracy of assays compared to bulk reactions, and have numerous applications in chemical screening, protein engineering, and next generation sequencing[1,2,3]. For example, digital droplet polymerase chain reaction (ddPCR) affords increased accuracy compared to bulk quantitative polymerase chain reaction (qPCR), with applications for genetic variation in cancers, detection of disease causing mutations, and prenatal diagnostics[4,5,6]. A challenge of droplet

microfluidics, however, is the requirement of microfluidic devices to partition samples; while microfluidics afford excellent control over droplet properties, they require specialized expertise to build and operate[7,8]. Consequently, droplet-based methods are largely limited to expert labs or, in rare instances, applications in which a commercial instrument is available[9,10]. To broaden the use of droplet assays, the requirement for specialized microfluidic instrumentation is a hurdle that must be overcome.

In this article, we describe Particle Templated Emulsification (PTE), a microfluidic-free method for performing reactions in monodispersed droplets. In PTE, templating particles engulf the sample into droplets in carrier oil by simple vortexing (**Figure 1**). As the system mixes, the aqueous portion fragments into droplets of reducing size until the droplets contain single particles, at which point further fragmentation is not possible because it requires breaking the particles. The engulfed sample surrounds the particles as a shell in the droplets, thereby encapsulating any dispersed cells, reagents, or functional moieties (**Figure 1D**). Thus, PTE requires no equipment or expertise to perform droplet reactions beyond a common vortexer. Additionally, droplet generation takes seconds compared to minutes or hours with microfluidics, and the amount produced is proportional to the container volume, not device operation time, making it supremely scalable. These benefits make PTE ideal for conducting droplet assays in a variety of circumstances in which microfluidics are impractical. Here, we demonstrate PTE and use it to conduct ddPCR.

[Place **Figure 1** here]

PROTOCOL:

1. Preparation of hydrogel particles for particle templated emulsification.

Hydrogel particles used for particle templated emulsification can be prepared using two different methods.

1.1. Preparation using commercially available particles

1.1.1. Add 0.5 g of dried polyacrylamide particles compatible with PTE (e.g., Bio-Gel P-60 Gel (Bio-Rad), 45-90 µm diameter) to 30 mL of sterile water in a 50 mL conical tube and mix well. Incubate at room temperature for 30 min.

1.2. Preparation using microfluidic fabrication of particles

NOTE: Polyacrylamide particles compatible with PTE can be prepared using commercially available drop makers (e.g., QX200 Drop Generator (Bio-Rad), RayDrop (Fluigent) etc.), or by custom microfluidic design.

1.2.1. Fabrication of custom master

1.2.1.1. Design a soft photolithography mask using computer-aided design (CAD)

software. Print the photomask with a 10 µm resolution on circuit board film.

1.2.1.2. Pour 1 mL of photoresist onto the center of a 3 in silicon wafer. Use a spin coater to create a 50 µm layer of photoresist by spinning it at 500 rpm for 30 sec followed by 1250 rpm for 30 sec. Place the wafer onto a hotplate set to 95 °C for 15 min to evaporate the solvent.

1.2.1.3. Secure the photomask onto the silicon wafer with a cover glass slide and expose the wafer under a collimated 190 mW, 365 µm UV LED for 2.5 min. Place the wafer on a hotplate set to 95 °C for 5 min for post exposure baking.

1.2.1.4. Develop the photoresist-silicon wafer by immersing it in a bath of 100% propylene glycol monomethyl ether acetate (PGMEA) for up to 15 min. Rinse the wafer with fresh 100% PGMEA followed by 100% isopropanol. Air dry the wafer.

1.2.1.5. Remove any residual isopropanol by drying the wafer on a hotplate set to 95 °C for 1 min. Place the wafer into a clean 3 in Petri dish.

1.2.2. Fabrication of the custom microfluidic device

1.2.2.1. Mix the polydimethylsiloxane (PDMS) silicon base and curing reagent in a 10:1 ratio by mass. Degas the mixed PDMS using a desiccator under house vacuum until no air bubbles are observable.

1.2.2.2. Pour the degassed PDMS over the master in the petri dish, ensuring the silicon wafer is completely submerged. Degas the silicon wafer and PDMS to remove any air bubbles that may have formed during pouring.

1.2.2.3. Cure the PDMS by placing the silicon wafer and PDMS into an oven set to 65 °C for at least 60 min. Excise a block of PDMS containing the microfluidic features from the petri dish using a scalpel. Take extra care to avoid damaging any features present on the silicon master.

1.2.2.4. Punch the inlets and the outlets into the PDMS block corresponding to the inlets and outlets in the microfluidic device using a 0.75 mm biopsy punch. Remove any dust and particulates with the repetitive application and removal of packaging tape to the surface of the PDMS block.

1.2.2.5. Clean a 50 mm x 75 mm glass slide by rinsing it with 100% isopropanol and subsequently air drying the surface. Plasma treat both the glass slide and the PDMS (features facing up) using 1 mbar of O_2 plasma for 1 min using a plasma bonder.

1.2.2.6. Affix the PDMS to the glass slide by placing the plasma treated PDMS with features facing down onto the glass slide, plasma treated side facing up. Place the slide into an oven set to 65 °C for at least 30 min to complete the bonding.

1.2.2.7. Treat all microfluidic channels with a fluorinated surface treatment to ensure surface hydrophobicity and prevent wetting. Bake the device at 65 °C for at least 10 min.

1.2.3. Fabrication of templating particles

1.2.3.1. Prepare a polyacrylamide (PAA) solution consisting of 6.2% acrylamide, 0.18% N,N'-methylenebis (acrylamide), and 0.3% ammonium persulfate. Load this solution into a 1 mL syringe with a 28G needle.

1.2.3.2. Prepare an insoluble continuous phase consisting of 5% (w/w) fluorosurfactant and 1% N,N,NN-tetramethylethylenediamine (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets. Load the solution into new 1 mL syringe.

1.2.3.3. Load both PAA and HFE solution containing syringes into syringe pumps (E.g., NE-501). Connect both syringes to the microfluidic device using polyethylene tubing inserted onto the syringe and into the device. Before the connecting, prime the pumps to remove the air from the tubing.

NOTE: Depending on the model, syringe pumps may be controlled with built in input, manufacture software, or a custom script (available at https://github.com/AbateLab/Pump-Control-Program).

1.2.3.4. Run the drop generation device with PAA and HFE oil inputs at 300 µL/h and 500 µL/h, respectively. Collect 1 mL of the droplets in a 15 mL collection tube and incubate for 3 h at room temperature for polymerization. After the incubation, remove the lower layer of oil by pipetting.

1.2.3.5. Add 1 mL of 20% (v/v) perfluoro-1-octanol (PFO) in HFE oil to the 15 mL collection tube as a chemical demulsifier. After mixing, spin down the 15 mL collection tube at 2000 x *g* for 2 min. Remove the PFO/HFE supernatant by pipetting. Repeat 1x.

1.2.3.6. Add 2 mL of 2% sorbitan monooleate in hexane to the 15 mL collection tube and vortex to mix. Spin the tube at 3000 x *g* for 3 min. Remove the supernatant by pipetting to remove surfactant/hexane solution. Repeat 2x.

1.2.3.7. Add 5 mL of TEBST buffer (20 mM Tris-HCl pH 8.0, 274 mM NaCl, 5.4 mM KCl, 20 mM EDTA, 0.2% Triton X100) and mix well. Spin down at 3,000 x *g* for 3 min. Remove the supernatant by pipetting. Repeat 3x.

1.2.3.8. Resuspend in 5 mL TEBST. This solution may be stored at 4 °C indefinitely.

2. Particle templated emulsification.

Following the preparation of templating particles, PTE is used to encapsulate the sample and

reagents in droplets.

2.1. Prepare the polyacrylamide particles for particle templated emulsification by centrifuging at 6000 x *g* for 1 min to pellet the particles, then remove the supernatant by pipetting and resuspend using sterile water. Repeat 3x to ensure removal of any residual TEBST.

2.2. Determine the concentration and diameter of the templating particles using a hemocytometer (or equivalent). Calculate individual particle diameter by measuring the diameters in pixels and converting to microns. Conversion of pixels to microns may be calculated using the hemocytometer (or equivalent) as a calibration slide and measuring the known grid distance in pixels.

2.3. Prepare the disperse phase in a fresh 1.5 mL microcentrifuge tube using a PCR master mix, the appropriate primers, and a fluorescein hydrolysis probe according to **Table 1**. Incubate at room temperature for 5 min under gentle agitation (10 rpm) using a tube rotator to ensure homogenous distribution of the components.

NOTE: The volume and target concentration of particles is based upon Poisson loading. As general rule, the number of particles should be an order of magnitude more than the number of samples to be encapsulated. For samples of unknown concentrations, a dilution series is necessary to ensure Poisson loading.

[Place **Table 1** here]

2.4. Centrifuge the disperse phase at 6000 x *g* for 1 min and remove the supernatant. Record the volume of the supernatant extracted and using the total disperse phase volume calculated in **2.3** determine the pellet volume.

NOTE: The amount of supernatant extracted will vary depending on particle packing, diameter, and concentration with a minimum expected volume of 300 µL.

2.5. Add 1 µL of 1.62 pg /µL *Saccharomyces cerevisiae* genomic DNA to the pellet from **2.4** and mix thoroughly by pipetting or vigorous tapping.

NOTE: The presence of excess aqueous content can decrease encapsulation efficiency. If the sample volume exceeds 1% of the pellet volume, concentrate the sample. If the sample cannot be concentrated, scale the PCR master mix and resulting pellet volume according to the sample volume. PTE permits the emulsification of small (10 µL) to large (2 mL) volumes of templating particles. The PCR master mix (**2.3**) and oil (**2.6**) can be scaled according to the target (**2.3**) and measured (**2.4**) volume of the particle pellet respectively.

2.6. Add 200 µL 2% fluorosurfactant in HFE oil to the tube as the insoluble continuous phase for emulsification. Ensure the pellet is dislodged by pipetting or tapping/flicking the tube. Then vortex at 3000 rpm for 30 sec.

NOTE: The setting corresponding to 3000 rpm may vary depending on brand and model.

2.7. Allow for the emulsions to settle for 1 min. Remove 100 µL of the bottom oil phase and replace this volume with fresh 2% fluorosurfactant in HFE oil. Gently invert the tube several times to mix. Repeat 3-5x or until small satellite droplets have been removed.

3. Digital droplet PCR and analysis.

3.1. After 2-5 min of settling, remove the bottom oil phase. Replace this volume with 5% fluorosurfactant in fluorocarbon oil (e.g., FC-40).

3.2. Using a wide bore pipette tip, carefully pipette the 100 µL of sample into 200 µL PCR tubes. Place the PCR tubes into a thermocycler and run according to **Table 2**.

[Place **Table 2** here]

3.3. Pipette the sample onto a counting slide using a wide bore pipette tip for fluorescent imaging. Image the sample using a fluorescent microscope with 490 nm excitation and 525 nm emission detection wavelengths.

3.4. Quantify the positive fluorescent droplets (N_p) and total drops (N_T) to verify the presence and calculate the number of template molecules (λ) using the fraction of positive droplets (N_p/N_T) and Poisson statistics:

$$\lambda = -\ln(1 - \frac{N_p}{N_T})$$

3.5. Calculate the 95% confidence interval (z_c = 1.96) by:

$$\lambda_{CI} = \lambda \pm z_c \sqrt{\frac{N_p}{N_T(N_T - N_P)}}$$

3.6. Calculate the sample concentration (molecules/µL) using the volume (ν in µL) of sample added in step **2.5**, using the equation given below. Determine the mean and standard deviation of the sample concentration using technical replicates.

$$concentration = \frac{\lambda}{\nu}$$

REPRESENTATIVE RESULTS:

[Place **Figure 2** here]

In PTE, the monodispersity of the emulsions is dictated by that of the templating particles, because the droplets have a diameter slightly larger than the particles. Thus, uniform particles are central to controlled PTE encapsulation[11]. A variety of methods exist for generating uniform templating particles, including chemical (sol-gel, emulsion polymerization), hydrodynamic (membrane emulsification, homogenization), and filtration methods. Microfluidic approaches in particular, afford superb monodispersity (**Figure 2A**) and allow additional particle engineering to enhance their functionality in PTE[12]. Alternatively, templating particles can be purchased, although their uniformity, while adequate, is typically less than with microfluidic generation[11].
To perform PTE, the particles are mixed with the sample to be encapsulated (**Figure 1A**), and the excess supernatant is removed by centrifugation and pipetting (**Figure 1B**), as illustrated by a photograph of a particle pellet at the bottom of a PCR tube (**Figure 2B**). The encapsulating oil containing a stabilizing surfactant is then added (**Figure 1C**), and the sample gently pipetted before vortexing for 30 seconds (**Figure 1D**), to generate the emulsion (**Figure 2C**). The resultant droplets contain a particle core and aqueous shell comprising the initial sample, within which reside the reagents, target molecules, and cells necessary for the reaction (**Figure 2D**). Just as in droplet microfluidic encapsulation, discrete entities like small beads or cells are encapsulated randomly and in accordance with a Poisson distribution, although nearly all droplets contain a templating particle due to the nature of PTE physics.

[Place **Figure 3** here]

Even in successful PTE, double or triple core droplets exist, though they generally contribute negligibly to the reaction, provided they are rare. Achieving a low frequency of multicore droplets while retaining adequate shells requires optimization of process parameters, including surface tension, inter-particle adhesion forces, sample viscosity, container size, and vortexing power and time. For example, a poorly optimized emulsification may contain polydispersed droplets with many templating particles (**Figure 3A**), indicating that the vortexing was insufficient to fully emulsify the sample. In such instances, detergents can be added to reduce inter-particle adhesion and lower surface tension, or vortexing power or time can be increased. Another common issue is generation of excessive satellites, which are small empty droplets (**Figure 3B**). Satellites can be unavoidable in PTE emulsions depending on the interfacial tension and rheological properties of the sample and carrier oil. However, they often result from not adequately removing excess sample prior to emulsification (**Figure 2B**), or vortexing with too much power, stripping the shells from the droplets. In a successful PTE emulsification, satellites should comprise no more than ~10% of the total encapsulated sample volume (**Figure 3C**)[11]. At this level, they usually contribute negligibly to the reaction and can be ignored. For aesthetic purposes, they can be cleared from the emulsion by washing with fresh oil (**Figure 3D**).

[Place **Figure 4** here]

To demonstrate the utility of PTE, we used it to perform microfluidic-free digital PCR[11]. Using the process, we encapsulated a sample comprising *S. cerevisiae* genomic DNA, and thermocycled it. In digital PCR, droplets containing amplified targets become fluorescent, while those without remain dim. Thus, a fluorescent droplet indicates a target, allowing direct quantitation of targets by counting positive droplets (**Figure 4A**). The number of fluorescent droplets thus scales with the target molecules, yielding few positives when the target is rare (**Figure 4B**) and many when it is abundant (**Figure 4C**). As with encapsulation of other discrete components, target encapsulation follows a Poisson distribution, allowing the positive droplet fraction to be transformed into the target concentration (**Figure 4D**), thereby demonstrating the ability to perform digital PCR with PTE[11].

[Place **Figure 5** here]

These results are repeatable using commercially available polyacrylamide particles (**Figure 5**) and demonstrate the ability of PTE to perform standard digital PCR with commercially available polyacrylamide particles, achieving accurate measurements over the same range.

FIGURES AND TABLES:

Figure 1. Overview of particle templated emulsification process. (**A**) Templating particles are mixed with reagents. (**B**) Excess reagents are removed following centrifugation. (**C**) The addition of template molecules occurs before the addition of oil. (**D**) Vortexing produces droplets containing a single template molecule. (**E**) Subsequent thermocycling and imaging allows for digital droplet analysis of target template.

Figure 2. Encapsulation of sample into droplets using particle templated emulsification. (**A**) templating particles used for particle templating emulsification. (**B**) Separation of templating particle pellet from supernatant following centrifugation. (**C**) Droplets resulting from particle templated emulsification with (**D**) identifiable aqueous shell.

Figure 3. Identification and cleanup of particle templated emulsification droplets. (**A**) Example of non-uniform droplet generation with multiple particles per droplet from insufficient vortexing. (**B**) Expected presence of satellites and droplets following particle templated emulsification and (**C**) the water-in-oil fractionation. (**D**) Resulting emulsion following oil washing. (**E**) Excessive satellite generation resulting from residual supernatant during particle templated emulsification.

Figure 4. Evaluation of particle templated emulsification digital droplet PCR. (**A**) Fluorescent imaging of the droplets identifies positive fluorescent droplets and negative non-fluorescent droplets. (**B**) Identification of rare template or low concentrations of template with digital droplet PCR. (**C**) Over abundant template encapsulation resulting in a variable number of template molecules per droplet.

Figure 5. Demonstration of digital droplet PCR using commercially available PAA. (**A**) Fluorescent imaging of the droplets identifies negative non-fluorescent droplets. (**B**)

Identification of low concentrations of template with digital droplet PCR. (**C**) Identification of high concentrations of template with digital droplet PCR.

Table 1. Preparation of the PCR master mix used with PTE for digital droplet PCR.

Table 2. Thermocycling conditions for digital droplet PCR using PTE emulsions.

DISCUSSION:

PTE uses particles to encapsulate samples in monodispersed droplets by vortexing. In addition to its simplicity and accessibility, PTE provides several additional benefits, including allowing large volumes of droplets to be generated instantaneously. Moreover, the process can be conducted in an isolated tube, obviating the need to transfer samples to microfluidic devices, streamlining the overall workflow and limiting opportunities for sample contamination or loss. The templating particles also provide a means by which to engineer the contents of the resultant droplet reactions. For example, particle size, chemistry, and wettability can be engineered for targeted biomolecule or cell capture, while functional moieties such as enzymes, actives, or nucleic acids, can be displayed on particle to facilitate reactions, such as for single cell sequencing or functional characterization. While the approach is flexible, there are nevertheless important constraints to its use. For example, it is not currently possible to perform droplet additions as are often conducted with microfluidics, requiring that all reaction components be introduced before encapsulation; this requires that reagents be compatible and stable until the droplets can be generated and, in the case of troublesome combinations, can often be addressed by quickly mixing and emulsifying the sample on ice. Alternatively, reactive components that can be triggered externally with light or heat can be used[13]. PTE thus provides a flexible and scalable method for conducting droplet assays accessible to non-experts. This, coupled with its innate simplicity and flexibility, makes PTE ideal for the execution and development of numerous droplet applications.

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DISCLOSURES:

Authors have nothing to disclose.

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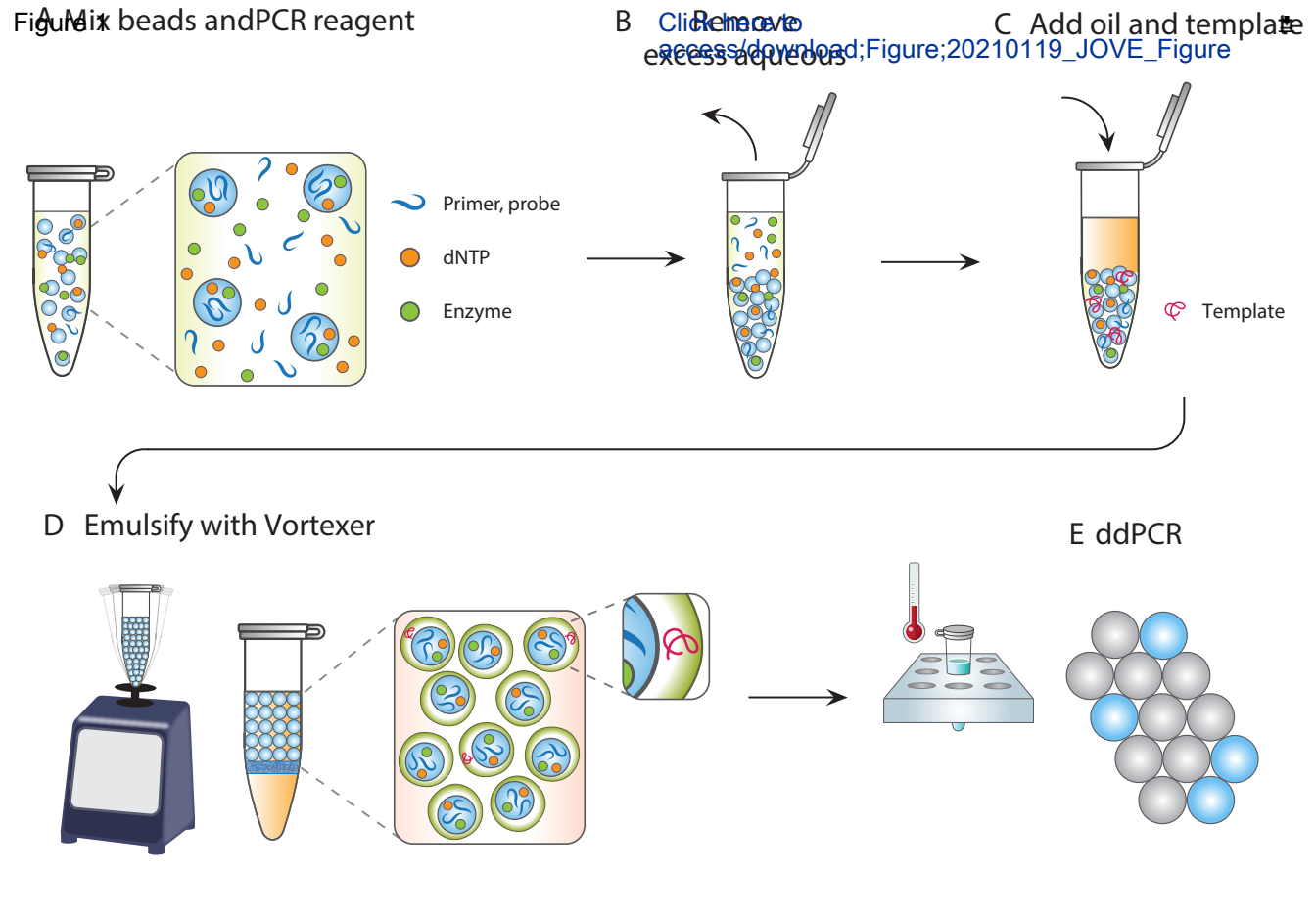

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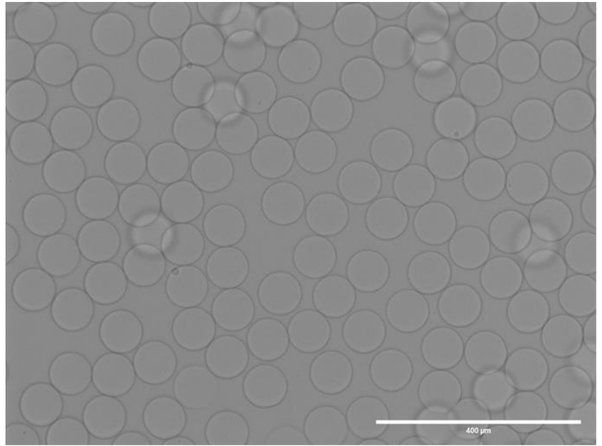

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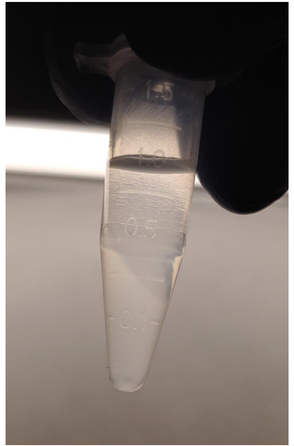

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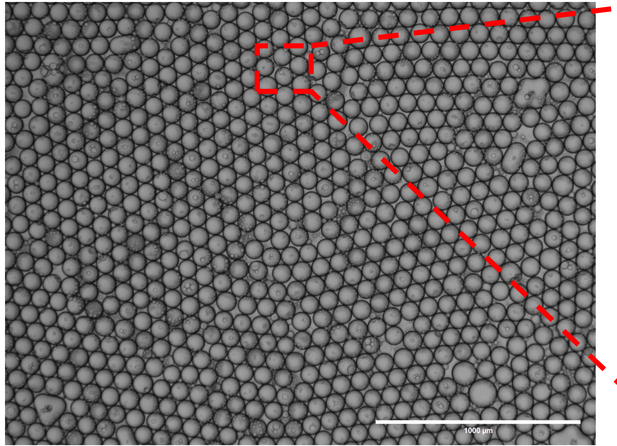

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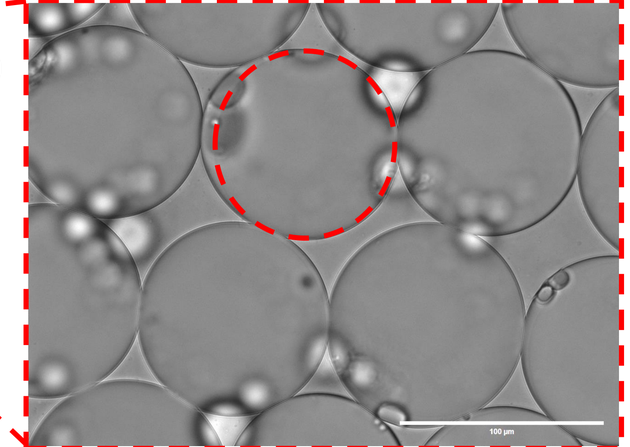

Figure 3

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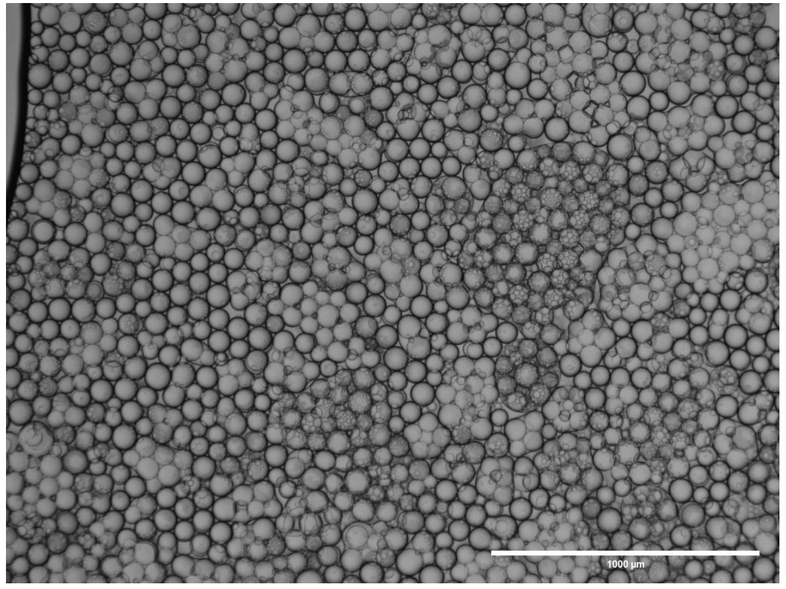

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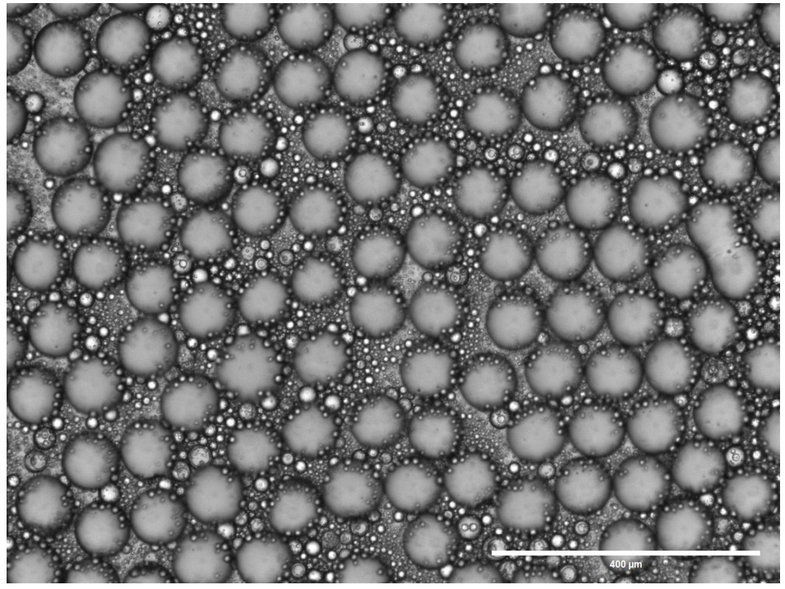

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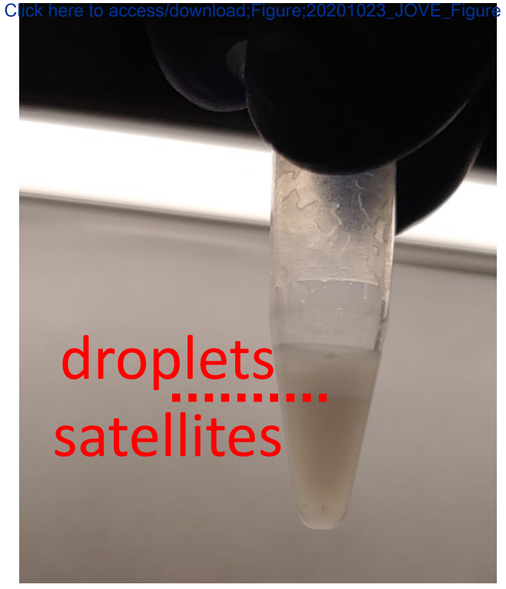

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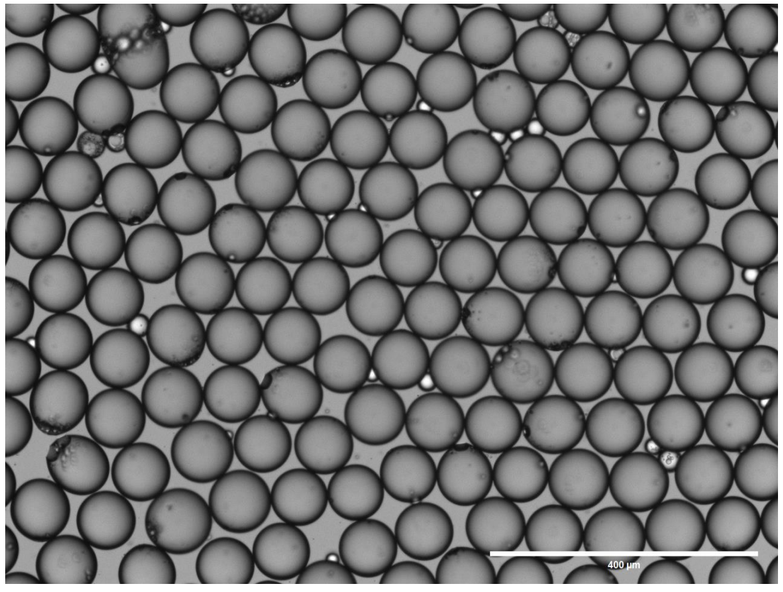

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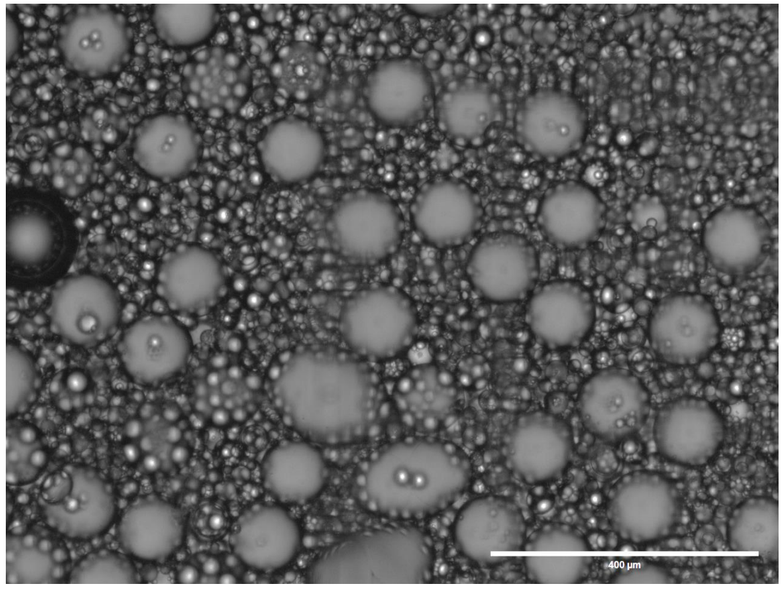

Figure 4

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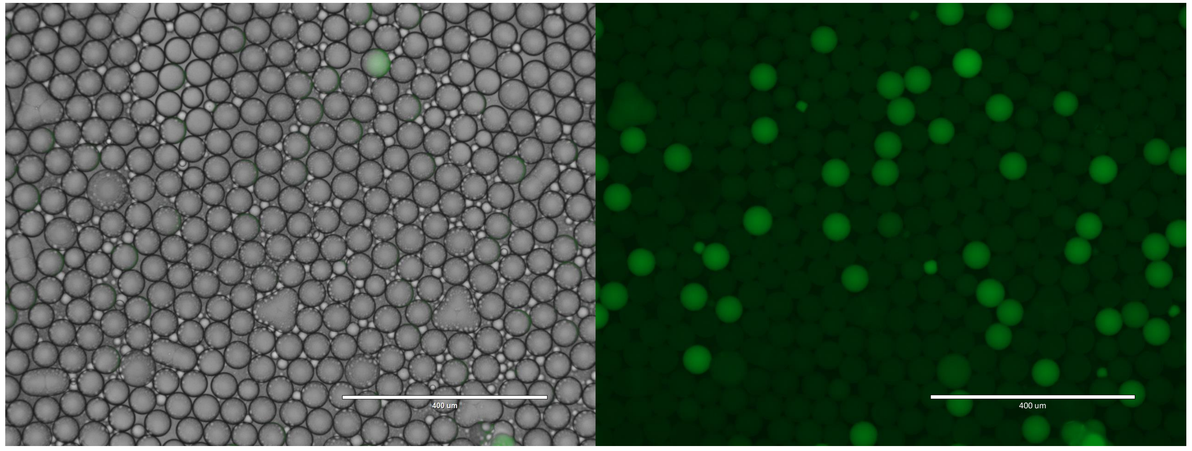

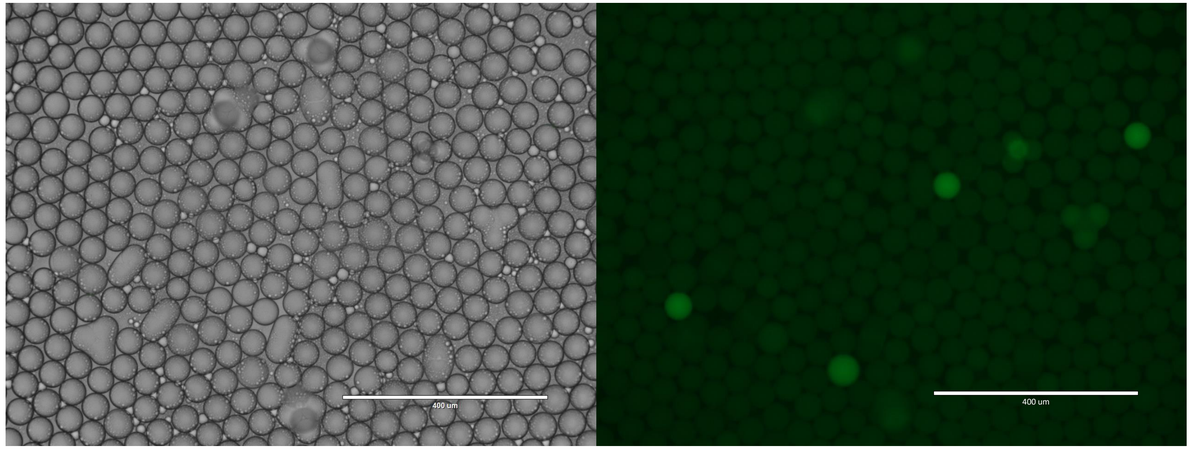

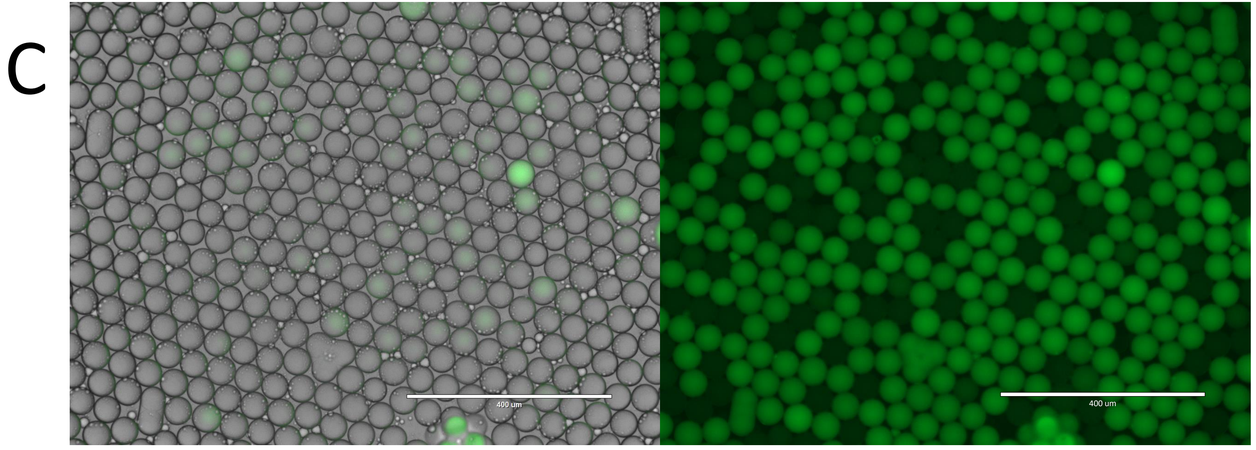

Figure 5

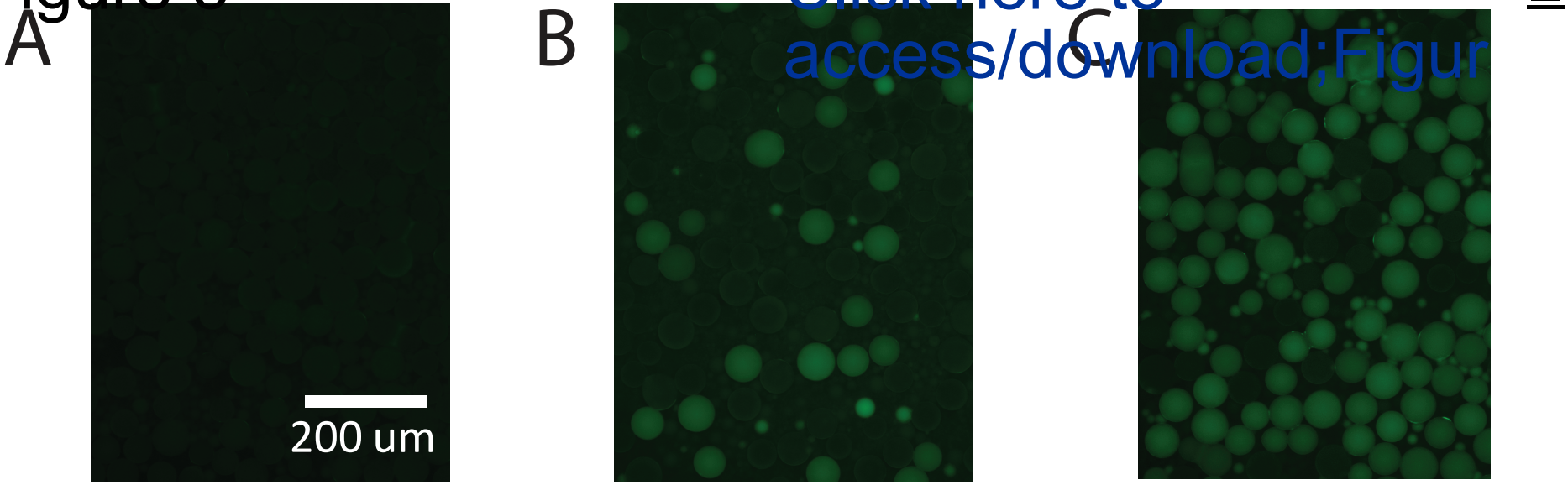

Table 1

Click here to access/download;Table;20210119_JOVE_Table 1.xlsx

Volume	Reagent
100 µL	Particles (450 particles / µL)
200 µL	2x PCR master mix
18 µL	10 µM forward primer
18 µL	10 µM reverse primer
18 µL	10 µM probe
0.8 µL	Triton X100
45.2 µL	Nuclease free water

Table 2

Click here to access/download;Table;20210119_JOVE_Table 2.xlsx

100 µL	Particles (450 particles / µL)
200 µL	2x PCR master mix
18 µL	10 µM forward primer
18 µL	10 µM reverse primer
18 µL	10 µM probe
0.8 µL	Triton X100
45.2 µL	Nuclease free water

Step	Temperatu	Dı	Notes
1	95 °C	2 min	
2	95 °C	30 s	
3	50 °C	90 s	
4	72 °C	60 s	
5			Repeat x34 steps 2 to 4
6	72 °C	2 min	
7	4 °C	hold	

Table of Materials

Click here to access/download;Table of Materials;20201023_JOVE_materials_v2.xlsx

Name	Company	Catalog Number
0.22 um syringe filter	Milipore Sigma	SLGP033RS
0.5M EDTA, pH 8.0	Thermo-Fisher	15575020
0.75 mm biopsy punch	World Precision Instruments	504529
1 mL syringes	BD	309628
1H,1H,2H-Perfluoro-1-Octanol (P	Sigma-Aldrich	370533
1M Tris-HCl, pH 8.0	Thermo-Fisher	15568025
27 gauge needles	BD	305109
3" silicon wafers, P type, virgin te	University Wafers	447
3D-printed centrifuge syringe hol	(custom)	(custom)
Acrylamide solution,40%, for electrophoresis, sterile-filtered	Sigma-Aldrich	A4058-100ML
Ammonium persulfate	Sigma-Aldrich	A3678-25G
Aquapel (fluorinated surface trea	Pittsburgh Glass Works	47100
Hexane	Sigma-Aldrich	139386
FC-40 fluorinated oil	Sigma-Aldrich	F9755
Isopropanol	Sigma-Aldrich	109827
N,N'-Methylenebis(acrylamide)	Sigma-Aldrich	146072-100G
NaCl	Sigma-Aldrich	S9888
Novec-7500 Engineering Fluid (HI	3M	98-0212-2928-5
polyethylene tubing	Scientific Commodities	B31695-PE/2
fluorosurfactant	Ran Biotechnologies	008-FluoroSurfactant
PGMEA developer	Sigma-Aldrich	484431
Photomasks	CadArt Servcies	(custom)
Platinum Multiplex PCR Master M	Applied Biosystems	4464263
Spin coater	Specialty Coating Systems	G3P-8
Span 80 (sorbitane monooleate)	Sigma-Aldrich	s6760
SU-8 3025 photoresist	Kayaku	17030192
Triton X-100 (octylphenol ethoxy	Sigma-Aldrich	t8787
Tween 20 (polysorbate 20)	Sigma-Aldrich	p2287
Platinum Multiplex PCR Master M	Applied Biosystems	4464263
Yeast FWD	IDT	5'-GCAGACCAGACCAGAACAA
Yeast REV	IDT	5'-ACACGTATGTATCTAGCCGA
Yeast Probe	IDT	5'-/56-FAM/ATATGTTGT/ZEN
EVOS FL AUTO	Life Technologies	
EVOS LED Cube, GFP	Life Technologies	AMEP4651
SYLGARD 184 KIT 1.1 LB (PDMS b	Dow Corning	DC4019862
TEMED	Thermo Fisher	17919
Saccharomyces cerevisiae genom	Milipore	69240-3
Expanded plasma cleaner (plasm	Harrick Plasma	PDC-002 (230V)

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\ATAAC-3
/TCACTCGCGCCTGGG/3IABkFQ/-3’

Dear Editor,

We have taken this opportunity to address the editorial and reviewer comments. For your convenience we have summarized these responses below.

Please reference our responses directly below each comment and with *blue italicized text*, to the editorial and reviewer comments with relevant italicized text quoted ("…") with changes marked with red text.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have taken this opportunity proofread the manuscript and to correct several grammatical errors pointed out by the editor and reviewers.

2. Please provide an email address for each author.

We have supplied the email addresses of the three authors under the affiliations.
"Daniel.Weisgerber@ucsf.edu
Makiko.Hatori@ucsf.edu
adam@abatelab.org"

3. Please define all abbreviations before use. E.g. PAA, PDMS, TEMED

We have proofread the manuscript with extra attention for undefined abbreviations. We have now properly defined all abbreviations before use.

4. Please use the imperative tense in the protocol, as if telling someone how to do the technique. E.g. "Load solution in.." instead of "solution is loaded…".

We have proofread the protocol with particular attention towards using the imperative tense.

5. Line 72: "…tube and mix well." Instead of "..tube mixing well.".

We have corrected the sentence.
"Add 0.5 g of dried commercial particles to 30 mL of sterile water in a 50 mL conical tube and mix well."

6. Line 84: 50 µm layer/coat?

We have corrected this within the protocol.
"Use a spin coater to create a 50 µm layer of photoresist by spinning at 500 rpm for 30 sec followed by 1250 rpm for 30 sec."

7. Line 85: Secure with what? Please add details here.

We have clarified this within the protocol.
"3. Secure the photomask onto the silicon wafer with a cover glass slide and …"

8. Lines 89-91: What is the concentration of PGMEA, isopropanol used?

In both cases 100% PGMEA and isopropanol were used. We have clarified this within the protocol.

"Develop the photoresist-silicon wafer by immersing it in a bath of 100% propylene glycol monomethyl ether acetate (PGMEA) for up to 15 min. Rinse the wafer with fresh 100% PGMEA followed by 100% isopropanol."

9. Lines 95-99: Please add details about degassing.

We have provided additional details regarding the procedure of degassing the PDMS within the protocol.

"Degas the mixed PDMS using a desiccator under house vacuum until no air bubbles are observable."

10. Line 107: Which tape is used?

We use Scotch tape for the removal of dust/debris from the microfluidic device prior to bonding. We have clarified this within the protocol.

"Remove any dust and particulates with the repetitive application and removal of packaging tape to the surface of the PDMS block."

11. Line 117: Please specify which fluid is used.

For the treatment of microfluidic devices we use Aquapel. However, Aquapel is a company/product. In an attempt to avoid commercial language (Editorial comment 19) we have rewritten the sentence to avoid confusion.

"10. Treat all microfluidic channels with a fluorinated surface treatment.
11. Bake the device at 65 °C for at least 10 min."

12. Line 125-126: "..and 1%" of what? Also specify if the syringe mentioned (line 126) is different from the one in line 124.

We have updated the protocol to include the name TEMED, 1% TEMED, and appropriately defining the abbreviation. We also specify that a new syringe should be used in loading this mixture.

"2. Prepare an insoluble continuous phase consisting of 5% (w/w) deprotonated fluorosurfactant and 1% N,N,NN-Tetramethylethylenedi (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets. Load the solution into new 1 mL syringe."

13. Line 133: "… droplets in.." instead of "…droplets to.."

We have updated the organization of this sentence as suggested.

"Collect 1 mL of the droplets in a 15 ml collection …"

14. Line 134-135: How and when is TEMED introduced?

TEMED was introduce within the oil phase (see Editorial comment 12). This describes the method of introducing TEMED for the polymerization of the PAA particles.

15. Line 136: How is the oil removed?

Within the protocol, we have clarified that the oil is removed using a pipette.

"Remove the lower layer of oil by pipetting."

16. Line 137: When should this be added? To which tube?

We have clarified which tube the PFO should be added, the collection tube now referenced as such within the previous step.

"Collect 1 mL of the droplets in a 15 ml collection tube and …"

"Add 1 mL of 20% (v/v) perfluoro-1-octanol (PFO) in HFE oil to the 15 mL collection tube as a chemical demulsifier."

17. Lines 141, 145, 152, 171: How are the supernatants removed? Using what?

Here we remove the supernatants using a pipette. We have updated the protocol to specify this.

"Remove the PFO/HFE supernatant by pipetting."

"Remove the supernatant by pipetting to remove surfactant/hexane solution."

"Remove the supernatant by pipetting."

"… remove the supernatant by pipetting and resuspend…"

18. Line 156: How is the average diameter determined? Please add details about microscopy/imaging.

The average diameter is determined using an optical microscope in correspondence with a calibration slide with known distances, necessary to convert pixels to distance. We have included a brief explanation of this process within the protocol.

"Calculate individual particle diameter by measuring the diameters in pixels and converting to microns. Conversion of pixels to microns may be calculated using the hemocytometer (or equivalent) as a calibration slide and measuring the known grid distance in pixels."

19. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead (E.g. Line 158: "Platinum Multiplex Master Mix" can be replaced by "PCR master mix").

We have proofread the manuscript and replaced the appropriate text with generic terms to avoid commercial language. We have noted any conflicts with editorial/reviewer comments within our response to reveiwers.

20. Line 159: What is the disperse phase prepared in?

We have clarified that the disperse phase is prepared in a fresh 1.5 mL microcentrifuge tube.

"prepare the disperse phase in a fresh 1.5 mL microcentrifuge tube according to:"

21. Line 169: Incubation temperature? Agitation speed? Instrument/method used for agitation?

We have identified the incubation temperature as room temperature and the agitation speed as 10 rpm using a tube rotator. The protocol has been updated accordingly.

"Incubate the disperse phase at room temperature for 5 min under gentle agitation (10 rpm) using a tube rotator to ensure homogenous distribution of the components."

22. Line 174: "..pellet from 2.5.." instead of "..pellet from 2.4.."? Please check.

We have checked and clarified that the pellet is from step 2.5, not 2.4.

"… pellet from 2.5 and mix thoroughly via pipetting …"

23. Line 175: "..excess aqueous content/phase.." instead of "..excess aqueous..".

We have updated the protocol to aqueous content instead of just aqueous.

"The presence of excess aqueous content can decrease encapsulation efficiency."

24. Line 203-205: Please provide details about fluorescent imaging (instruments used, wavelengths etc.). Also add these details in the table of materials.

We use an EVOS FL Auto Imaging System, because the instrument name includes commercial language we have defaulted to a generic fluorescent microscope as suggested (Editorial comment 19). We have included the appropriate excitation and emission for fluorescent imaging.

"Image the sample using a fluorescent microscope with 490 nm excitation and 525 nm emission detection."

25. Please include the details from Step 1 (preparation of the particles, fabrication of the device) in the highlighted part that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have highlighted additional portions of the protocol that details the preparation of the microfluidic device and templating particle fabrication.

26. Please consider providing reaction set-ups and solution compositions as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

As suggested, we have converted the Tables to separate .xlsx files and uploaded them within the Editorial Manager.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper entitled "Particle templated emulsification enables microfluidic free droplet assays" is very interesting and significant to lower the access barrier to droplet-based digital PCR. However, the paper lacks some specifics to be truly reproducible by a non-expert. In addition, the paper lacks of a method to analyze fully the dPCR results. Also, it does not discuss the limitations of using commercially available particles due to their non-uniformity on the results of dPCR.

We have expanded upon the analysis of the dPCR results, included results from commercially available particles, and also cited previous work (ref 11) that provides additional information on dPCR analysis and commercial limitations.

Specific remarks:

- Figure 1 does not show when the sample is added, panel A only describes the PCR mix

We have updated Figure 1 to include where/when the sample is added.

- line 71: the authors should provide the dimensions of the commercially available particles

We have updated the text to include dimensions of the commercially available particles.

“Commercially available polyacrylamide particles compatible with particle templated emulsification include Bio-Gel P-60 Gel (Bio-Rad), 45-90 µm diameter.”

- line 117: what is the fluorinated surface treatment specifically?

The fluorinated surface treatment used to treat our devices is Aquapel. As requested (Editorial comments 18), we have avoided directly identifying the fluorinated surface treatment as Aquapel since it includes unpublishable commercial language. We have clarified within the materials list Aquapel as the fluorinated surface treatment.

“Treat all microfluidic channels with a fluorinated surface treatment to ensure surface hydrophobicity and prevent wetting.”

- line 125: what is deprotonated Krytox 157 FSH specifically?

Krytox (157 FSH) is an ionic surfactant used in forming droplets. The surfactant is necessary for stabilizing drops produced either microfluidically or using particle templated emulsification. Specifically, the Krytox surfactant consists of a functionalized carboxylic acid group at the terminal fluoromethylene group of poly(hexafluoropropylene oxide).

However numerous alternative surfactants can be used within this protocol. We have updated the protocol to use only one surfactant along with a brief explanation of the role in droplet formation.

“Prepare an insoluble continuous phase consisting of 5% (w/w) 008-fluoroSurfactant and 1% N,N,NN-Tetramethylethylenedi (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets.”

- line 130: define the acronym PAA

We have updated the text accordingly to define PAA.

“Prepare a polyacrylamide (PAA) solution consisting of 6.2% acrylamide, 0.18% N,N’-methylenebis (acrylamide), and 0.3% ammonium persulfate. Load this solution into a 1 mL syringe.”

- line 131: what syringe pumps are used? Is a custom script necessary, that sounds over-specific to the system used by the authors

We use New Era NE-501 syringe pumps in our setup, which we have now identified in the protocol. These pumps are controlled using a PC using a custom script we have made available using GitHub (see link). However, this is not necessary as many alternative commercially available syringe pumps do not require the use of the script. Within this step we have identified the critical flow rate requirement that must be met regardless of the syringe pump.

“Load both PAA and HFE solution containing syringes into NE-501 syringe pumps.”

“Note: Depending on the model, syringe pumps may be controlled with built in input, manufacture software, or for NE-501 pumps a custom program (available at https://github.com/AbateLab/Pump-Control-Program).”

- line 134: when is TEMED introduced and how? There is no mention of TEMED before

We have updated the protocol to define TEMED and indicate where it is introduced.

“Prepare an insoluble continuous phase consisting of 5% (w/w) fluorosurfactant and 1% N,N,NN-Tetramethylethylenedi (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets.”

- line 146: how long could the beads be stored at 4C?

The polyacrylamide beads can be stored indefinitely at 4C. We have updated the protocol to specify this fact.

"Resuspend in 5 mL TEBST. This solution may be stored at 4°C indefinitely."

- line 173: the authors should discuss the importance/impact of the pellet volume, and how that step may affect the results

One particular strength of particle templated emulsification is the ability to scale the reaction. The reaction described within this protocol targets 100 uL but can be expanded to larger (1 mL) or smaller (10 uL) pellets with the PCR master mix and oil being scaled accordingly. We have expanded on this explanation within the protocol.

"NOTE: Particle templated emulsification permits the emulsification of small (10 uL) to large (1 mL) volumes of templating particles. The PCR master mix (step 2.3) and oil (step 2.6) can be scaled according to the target (step 2.3) and measured (step 2.5) volume of the particle pellet respectively."

- line 179: define "weitz", define HFE oil

We have corrected weitz with the product name and defined HFE oil more accurately. The HFE oil is Novec 7500 Engineering Fluid from 3M. As requested, we have used a generic name to avoid commercial language (Editorial comment 19).

"Add 200 µL 2% 008-fluoroSurfactant in HFE oil to the tube as the insoluble continuous phase for emulsification."

"2. Prepare an insoluble continuous phase consisting of 5% (w/w) 008-fluoroSurfactant and 1% N,N,NN-Tetramethylethylenedi (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets"

- line 209: provide the formulation to calculate the statistical error

We have expanded on the calculations used within the protocol to include statistical error and concentration.

We have added in corresponding calculation to determine the statistical error.

"The 95% confidence interval (z_c = 1.96) for this calculation is given by:

$$\lambda_{CI} = \lambda \pm z_c \sqrt{\frac{N_p}{N_T(N_T - N_P)}}$$

"

- line 263: Figure 4 does not demonstrate the ability to perform digital PCR, you should cite your work in ref. 11

The images presented in Figure 4 are the fluorescent images following digital droplet PCR. The positive (green) droplets fluoresce due to the amplification of the yeast genome using a TaqMan probe within these droplets. The negative (blank) droplets, also containing the TaqMan probe, do not fluoresce because there is no amplification occurring within those droplets. Because the drops either fluoresce or

do not fluoresce, the result is digital removing ambiguity from the result. Further analysis of the We have updated the manuscript to include additional references to the work, ref. 11.

- in the material list:
There is a difference between manufacturer and supplier that does not appear in the table: for instance, Sylgard 184 silicone elastomer kit is manufactured by Dow Chemicals and can be purchased at Krayden (or somewhere else).
There is no reference for TEMED, yeast genomic DNA, Tris-hcl should Tris-HCl, Microchem is now called Kayaku, HFE 7500 is referenced as HFE in the text or Novec 7500 in the list of material, Aquapel is not explicitly used in the protocol, the surfactant is appropriately called PEG-PFPE surfactant when it is called weitz in the protocol

We have updated the material list and protocol to affect these changes.

Reviewer #2:
Manuscript Summary:
This paper describes a facile method to produce monodisperse monodisperse droplets by bulk vortexing. Digital PCR, although it was only qualitatively, was performed to demonstrate its application. It was interesting to know digitalized PCR can be performed in such a way.
Although this protocol promises to improve the availability of digital droplet assays, I see that there are many limitations users must take care. I think it is very important to clarify the extent of applicability of this method. In addition, the structure of the paper is not carefully examined. I recommend authors to restructure the whole story and re-submit the paper.

This paper was purposely structured to fulfill the unique format JOVE provides scientists and is based upon the resources made available to the authors.

Major Concerns:
1. Authors claimed that this method is advantageous because it does not require microfluidics. However, in the results section, they only described the results with gel beads produced by microfluidics. Authors should explain the results with the commercial beads, such as Bio-Gel beads mentioned by authors, and compare the effect of uniformity.

We have prepared an additional Figure 5 with additional ddPCR data using the commercial beads and addressed the effect of uniformity within the manuscript pertaining to this discussion. More information regarding this topic can also be found within reference 11.
"These results are repeatable using commercially available polyacrylamide particles (**Figure 5**). This demonstrates the capability of PTE to perform standard digital PCR with commercially available polyacrylamide particles, achieving accurate measurements over the same range."

2. In my understanding authors assumed that components of reaction (enzymes, primers, etc.; Fig. 1A) is uniformly distributed inside and outside of gel beads. However, I am not sure if it is really the case. Since most of the mixture are removed at steps B to C in the same figure, I believe knowing the concentration of reaction inside the gel beads is important. Authors also should clarify if there is a possible inhibition of the reaction due to the presence of gel.

The polyacrylamide hydrogels can be prepared with adjustable pore sizes allowing for varying degrees of reagent and/or sample penetration depending on the given application. Following the removal of excess aqueous content/phase in step B, a significant amount of aqueous content/phase remains within the interstitial spaces of the templating particle pellet. After emulsification, this volume results in the aqueous shells of each droplet. The template DNA, also within this volume, can then interact with any reagents within the droplet for digital droplet PCR. Previous work has investigated the possible inhibition of the PCR reaction using polyacrylamide. We feel that addressing this within the text, with supporting data, is outside the scope of the protocol and instead reference the work establishing particle templated emulsification.

3. Authors assume that (and I agree that) the template DNA is present only in the thin aqueous layer at the surface of the beads. I wonder how authors can estimate the volume of this layer, which should be required to estimate the absolute concentration of the template. Authors described only a single equation in page 6, but there is no quantitative comparison between the theory and experiment.

The volume of this layer has can be optically evaluated to determine the volume of the aqueous shell. The determination of template concentration using this information is overly complicated and prone to inaccuracies. Another, more accurate determination of the template concentration can be calculated using the Poisson statistics mentioned from page 6. Using the fraction of positive droplets (number of positive droplets / negative droplets) the number of template DNA can be determined. Knowing the volume of sample added (1 uL in this example) then allows for a determination of the concentration (molecules / volume) of template DNA to be determined. We have updated the protocol to better reflect this analysis. A more quantitative analysis of this methodology, including a comparison to standard microfluidic digital PCR and dilution curves is covered in previous work. We have included additional references to reflect this.

"Quantify the positive fluorescent droplets (N_p) and total drops (N_T) to verify the presence and calculate the number of template molecules (λ) using the fraction of positive droplets (N_p/N_T) and Poisson statistics:

$$\lambda = -\ln(1 - \frac{N_p}{N_T})$$

The 95% confidence interval ($z_c = 1.96$) for this calculation is given by:

$$\lambda_{CI} = \lambda \pm z_c \sqrt{\frac{N_p}{N_T(N_T - N_P)}}$$

Calculate the sample concentration (molecules/µL) using the volume (v in µL) of sample added in step **2.6**, the concentration of the sample can be calculated by:

$$concentration = \frac{\lambda}{v}$$

The mean and standard deviation of the sample concentration is determined using technical replicates."

Minor Concerns:

4. It is better to explain what are HFE-7500 and perfluoro-1-octanol, their physical properties, and how they work in the protocol. Not all authors are familiar with these materials.

We have briefly elaborated on the purpose of these chemicals within the protocol to benefit users.

"Prepare an insoluble continuous phase consisting of 5% (w/w) 008-fluoroSurfactant and 1% N,N,NN-Tetramethylethylenedi (TEMED) in hydrofluoroether (HFE) oil for the generation and stabilization of droplets."

"Add 1 mL of 20% (v/v) perfluoro-1-octanol (PFO) in HFE oil to the 15 mL collection tube as a chemical demulsifier."

5. In line 161, authors recommended the particle concentration should be 450 particles/uL. I wonder how this concentration is important, and how it may be affected by the size of beads.

The protocol is flexible in regard to the particle concentration. The target is to supply sufficient templating particles for the Poisson loading of the sample. We have clarified this fact within the protocol.

"NOTE: The presence of excess aqueous content can decrease encapsulation efficiency. If the sample volume exceeds 1% of the pellet volume, concentrate the sample. If the sample cannot be concentrated, the PCR master mix and resulting pellet volume can be scaled according to the sample volume."

6. In line 171, authors noted that it is important to record the volume of removed supernatant. It is informative to mention the typical volume removed in this protocol.

We have updated the protocol to better explain the expected volume of supernatant that should be removed.

"NOTE: The amount of supernatant extracted will vary depending on particle packing, diameter, and concentration with the minimum expected volume is 300 µL."

7. Line 174: The word "aqueous" is adjective, not noun.

We have corrected the error with the addition of a noun.

"… excess aqueous content *…"*

8. Line 175 to 177: The sentence is weird. I recommend to rewrite something like this,

Note: Presence of excess aqueous phase often deteriorate the singe-bead encapsulation. If the sample volume exceeds 1% of the pellet volume, the excess aqueous phase should be removed by a second centrifugation at 6000 x g for 1 min, after mixing the sample and the pellet.

We have rewritten the sentence to improve its clarity.

"NOTE: The presence of excess aqueous content can decrease encapsulation efficiency. If the sample volume exceeds 1% of the pellet volume, concentrate the sample. If the sample cannot be concentrated, the PCR master mix and resulting pellet volume can be scaled according to the sample volume."

9. Line 179 to 184: What is 2% "weitz"?

We have better defined the continuous phase within the protocol.

“Add 200 µL 2% 008-fluoroSurfactant in HFE oil to the tube as the insoluble continuous phase for emulsification.”

10. Line 183: Remove this or the.

We have updated the protocol accordingly.

“Gently invert the tube several times to mix.”

11. Line 183 to 184: It is unclear which step should be repeated for 3 to 5 times.

We have separated this instruction into its own step referencing the previous step to better clarify what is to be repeated.

“Repeat step **2.8** up to 3-5 times or until small satellite droplets have been removed.”