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Combinatorial co-culturing and amplicon sequencing (Cocoa-seq) v1.0

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Protocol status: Working
This protocol may need to be cleaned up, but the main components are there.

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Disclaimer

Abstract

While synthetic ecology approaches provides phenotypic observations on how interactions
change in different contexts and contribute to overall community function through experimentation by constructing and interrogating defined co-cultures of cultured representatives, these approaches cannot be applied to systems where relevant isolates are not available or for specific natural strain diversity. To synthesize the reductionism of synthetic ecology while embracing natural diversity, we developed Cocoa-seq (combinatorial co-cultivation and amplicon sequencing), a microfluidic workflow utilizing the high throughput nature of nanoliter-scale, water-in-oil droplets to generate and profile co-cultures generated by the stochastic co-encapsulation of cells from samples, circumventing laborious axenic isolation. The workflow multiplexes over a thousand 16S amplicon libraries from droplet co-cultures into one sequencing library, with potential for even higher scalability. This protocol details steps to generate and process droplet co-cultures, adapt commercially available barcoded hydrogel beads to include the 16S v4 forward primer, and enable droplet-enabled co-culture multiplexing into one library. In an accompanying manuscript, we benchmarked Cocoa-seq with a model synthetic co-culture as well as mock communities of varying rank abundances and found that beta-diversity distributions obtained with Cocoa-seq qualitatively recapitulate expectations.

Purchase of barcode hydrogel beads (BHB) and related oligos

1 Purchase CustomSeqReady beads (inDrop variation, BD ca. 147k) from RAN Biotechnologies (**https://www.ranbiotechnologies.com/catalog/surfactants-and-beads/**) (Prices will change, but about $1000/million beads (~250 µL of packed beads)). Beads are stable at 4 C for up to 6 months, protected from light. (Verified with QC hybridization probes. Although we did not test beyond this time period.)

These barcode beads can also be generated (protocol for HyDrop variation to be up soon). Requires microfluidic droplet generation to generate polyacrylamide beads and performing a long split and pool barcode sequencing protocol.

2 Purchase relevant oligos required for BHB primer extension/QC, droplet PCR, and library preparation from IDT: list of oligos to order.xlsx 12KB

Buffer preparation

3 Prepare the following buffers:

Hydrogel bead wash (HWB) buffer

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	49 mL		
	Tris HCl pH 8.0	250 µL	1 M	0.005 M
	EDTA	500 µL	0.5 M	0.005 M
	Tween 20	250 µL	10% (v/v)	0.05%
	Total	50 mL		

STOP-25 buffer

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	44.835 mL		
	Tris HCl pH 8.0	500 µL	1 M	0.01 M
	EDTA	2.5 mL	0.5 M EDTA	0.025 M
	Tween 20	500 µL	10% (v/v)	0.1%
	KCl	1.67 mL	3 M	0.1 M

	A	B	C	D
	Total	50 mL		

STOP-10 buffer

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	46.08 mL		
	Tris HCl pH 8.0	500 µL	1 M	0.01 M
	EDTA	1.25 mL	0.5 M	0.0125 M
	Tween 20	500 µL	10% (v/v)	0.1%
	KCl	1.67 mL	3 M	0.1 M
	Total	50 mL		

TET buffer

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	48 mL		
	Tris HCl pH 8.0	500 µL	1 M	0.01 M
	EDTA	1 mL	0.5 M	0.01 M
	Tween 20	500 µL	10% (v/v)	0.1%
	Total	50 mL		

Neutralization solution

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	42.5 mL		
	Tris HCl pH 8.0	5 mL	1 M	0.1 M
	EDTA	1 mL	0.5 M	0.01 M
	Tween 20	500 µL	10% (v/v)	0.1%
	NaCl	1 mL	5 M	0.1 M
	Total			

QC buffer

	A	B	C	D
	Component	Amount	Stock concentration	Final concentration

	A	B	C	D
	Molecular grade H2O	32.33 mL		
	Tris HCl pH 8.0	250 µL	1 M	0.005 M
	EDTA	500 µL	0.5 M	0.005 M
	Tween 20	250 µL	10% (v/v)	0.05%
	KCl	16.67 mL	3 M	1 M
	Total			

Hybridization buffer

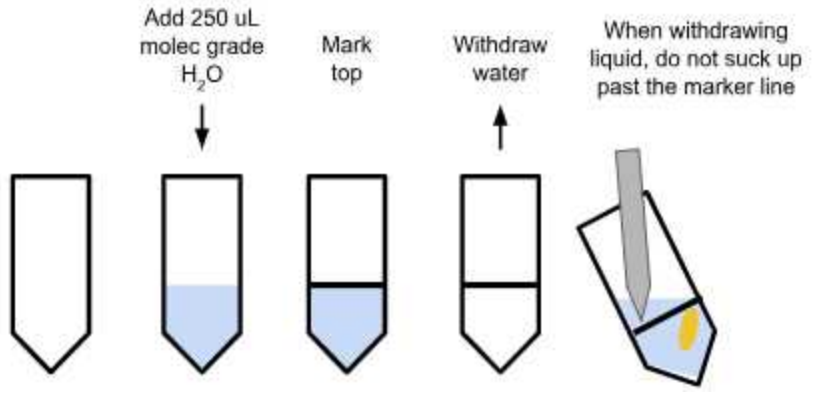
	A	B	C	D
	Component	Amount	Stock concentration	Final concentration
	Molecular grade H2O	43.49 mL		
	Tris HCl pH 8.0	500 µL	1 M	0.01 M
	EDTA	10 µL	0.5 M	0.0001 M
	Tween 20	500 µL	10% (v/v)	0.1%
	KCl	5.5 mL	3 M	0.33 M
	Total	50 mL		

For all of the above, 0.2 µm filter and store at room temperature for 6 months.

BHB primer extension

4 Working with hydrogel beads can be difficult to their translucence, making it difficult to see the interface between bead pellets and the supernatant after centrifugation. To ensure proper handling, use tubes that are easy to see through. If you have trouble seeing the boundary between pelleted beads and the supernatant, try a variety of backgrounds or check different angles – the boundary is the most distinct when viewing the slanted interface towards yourself at the bottom of a 1.5 mL microcentrifuge tube, but can be seen in a 2 mL tube as well.

4.1 If you have difficult viewing the pellet-supernatant interface, don't try to remove as much supernatant as possible and accidentally suck up beads. Use a sharpie to mark all microcentrifuge tubes you are using at the 250 uL volume with 250 uL molecular grade water and then remove the water with a µ-pipette. When taking out supernatant, do not remove liquid past the 250 uL line and pipette supernatant slowly. Since we are doing three washes for each wash, not all supernatant needs to be removed.



Marking tubes with. Credit to Erica Gardner (Lin Lab) for making this figure.

4.2 Also, pay attention to which side the BHB pellet leans towards after centrifugation. The BHB pellet will lean towards the end of the centrifuge tube facing out from the center of the centrifuge. This will help keep track of where to expect the pellet.

5 Turn on a heat block and set to 60 °C

6 Aspirate the 1 mL of inDrops BHB suspension from RAN Biotech with 1000 uL pipette tip 3-5 times to mix.

7 For two 1.5 mL (marked, if you are using that to prevent bead removal) centrifuge tubes each, mix 500 uL of the mixed inDrops BHB suspension and 1000 uL of hydrogel bead wash (HBW). Vortex well. Centrifuge at 1000 x g, Room temperature, 00:01:00 and remove supernatant. Do three total washes. Beads will swell so take precaution not to remove beads. 1m

8 With the 10X isothermal amplification buffer from the Bst 2.0 DNA polymerase kit, make 2 mL of 1X buffer (200 uL of 10X isothermal amplification buffer and 1800 uL of molecular grade water and vortex well). Add 1 mL to each tube. Centrifuge at 1000 rpm, Room temperature, 00:01:00 The bead pellet will have shrunk. Remove supernatant. 1m

9 For hybridization and elongation of the 515f region on the bead oligos, pool the following reagents in a **single** 1.5 mL microcentrifuge tube:

	A	B	C	D	E
	Component	Amount	Stock Concentration	Final concentration	Dilution
	10X isothermal amplification buffer	100 µL	10X	1X	10

	A	B	C	D	E
	Bst 2.0 DNA polymerase	43.7 µL	8000 U/mL	350 U/mL	22.9
	dNTPs mix	65 µL	10 mM each	650 µM each	15.4
	515f_customseqready oligo	100 µL	100 µM	10 µM	10
	BHB suspension - aspirate to mix first	x µL (should be around 500)			
	Molecular grade H2O	691.3-x µL			
	Total	1000 µL			

Aspirate by pipetting to mix

10 Incubate at 60 °C for 01:00:00 in the preheated heat block and cover with aluminum foil to protect from light 1h

10.1 If needed, this step can be held here for longer if you need to step out.

11 Aspirate by mixing and split the volume into 2 marked 1.5 mL microcentrifuge tubes again, for each add 1000 µL of STOP-25 buffer, rotate tubes at Room temperature for at least 00:30:00 30m

11.1 POTENTIAL PAUSE POINT: after this, BHBs can be stored at 4 C (fridge) overnight

12 Centrifuge at 1000 x g, Room temperature, 00:01:00, remove supernatant 1m

13 Wash with STOP-10 buffer three total times - each time add 1 mL STOP-10 buffer, cover microcentrifuge tube with aluminum foil, rotate for 15 min at Room temperature, 1000 x g, Room temperature, 00:01:00, and remove supernatant. 1m

14 Prepare fresh denaturation solution while you're waiting

	A	B	C	D	E
	Component	Amount	Stock concentration	Final concentration	

	A	B	C	D	E
	Molecular grade H2O	19.363 mL			
	NaOH solution	300 µL	10 M	0.15 M	
	Brij-35	336 µL	30% (wt/wt)	0.5%	
	Total	20 mL			

Filter through 0.2 µm sterile syringe filter with sterile packaged 10 mL syringe into a 50 mL Falcon tube

15 Wash with denaturation solution three times total:

15.1 First time: Add 1000 uL denaturation solution, rotate for 00:10:00 at Room temperature, centrifuge at 1000 x g, Room temperature, 00:01:00, discard supernatant 11m

15.2 Other times: Add 1000 uL denaturation solution, invert and flick, incubate for 00:01:00 at room temperature, centrifuge at 1000 x g, Room temperature, 00:01:00, remove supernatant 2m

16 Wash with neutralization buffer twice:

16.1 Add 1000 uL neutralization solution, invert and flick, incubate for incubate for 00:01:00 at room temperature, centrifuge at 1000 x g, Room temperature, 00:01:00, remove supernatant 2m

16.2 POTENTIAL PAUSE POINT: If stopping here, wash the HBs three times in 1000 uL TET buffer, typical vortex and centrifugation and store protected from light at 4 C. If not stopping here, just continue with the next step without TET buffer washes.

17 Wash three times with 1000 uL HBW buffer (beads will swell.) (Add 1000 uL HBW buffer to each tube, vortex to mix, centrifuge 1000g for 1 minute, and remove supernatant to 250 uL mark.) On last wash - before adding last round of 1000 uL hybridization buffer - transfer 10 uL of packed BHBs (from pellet) to a 1.5 mL microcentrifuge tube and add 90 uL of TET buffer. Label the tube “BHBs before cleanup” and store them at 4 C (fridge), protected from light.

18 Wash three times with 1000 uL hybridization buffer.(Add 1000 uL hybridization buffer to each tube, vortex to mix, centrifuge 1000g for 1 minute, and remove supernatant to 250 uL mark.)

19 Add 62.5 uL 100 uM 515f RC oligo to 250 uL bead suspension (to 20 uM oligo final concentration), mix well by aspirating with pipette tip

20 Rotate at room temp, protected from light for 30 minutes

21 Add below to each tube:

	A	B	C	D	E	F
	Component	Amount (x=total amount of bead suspension)	Amount (if x = 312.5 µL)	Stock concentration	Final concentration	Dilution
	10X ExoI buffer	0.2x µL	62.5 µL	10X	1X	10
	ExoI	0.027x µL	8.4 µL	20 U/µL	0.27 U/µL	74.07
	Water	0.773x µL	241.6 µL			
	Total volume of hybridization buffer/oligo mix	x µL	312.5 µL			
		2x µL	625 µL			

22 Rotate the tubes for 2 hours at room temperature, protected from light

23 Add 1000 uL STOP-25 buffer, invert a few times, centrifuge at 1000 x g, Room temperature, 00:01:00 , remove supernatant

24 Wash with STOP-10 buffer three times - each time add STOP-10 buffer to 1 mL, rotate for 15 min at room temperature, centrifuge, and remove supernatant

25 Wash with denaturation solution three times, as per Step 15.

26 Wash with neutralization buffer twice, as per Step 16.

27 Wash three times in TET buffer (1 min at 1000g), remove supernatant, combine to one 1.5 mL microcentrifuge tube and store at 4 C, protected from light.

Quality control of primer-extended BHBs

28 To check bead barcodes for 16S addition, use FAM oligo probes (100 uM each, 0.22 filtered, use column filters) (515f-FAM and PE1-FAM). Defrost in dark (covered ice bin with ice is fine)

29 There will be 2 (for “before” and “after” clean-up) x number of BHB replicate tubes = total tubes to label. Label 1.5 mL microcentrifuge tubes for each condition. Label the 60 uL volume line with molecular grade water and a Sharpie and remove the molecular grade water for each tube.

30 Centrifuge the “Before ExoI clean up” tube(s) and finished bead tube(s) at 1000 g for 1 min, remove most supernatant without disturbing bead pellet. Grab 5 uL of close-packed BHBs from all tubes and put into new 1.5 mL microcentrifuge tubes for each condition. Refill the “Before ExoI clean up” and “finished BHBs” tube with 1000 uL TET buffer.

31 Add 1.4 mL QC buffer to the 5 uL of close-packed HBs beads

32 Vortex mixture, centrifuge at 1000 g at 1 min and remove supernatant so the remaining total volume of QC buffer is 60 uL in each tube. Aspirate with pipette tip to mix.

33 Prepare two new 1.5 mL tubes (PE1 and 515f) for each tube you current have, add 27 uL of respective bead suspension to each.

34 Add 3 uL of each respective 100 uM FAM-oligos to each tube (10 uM final concentration).

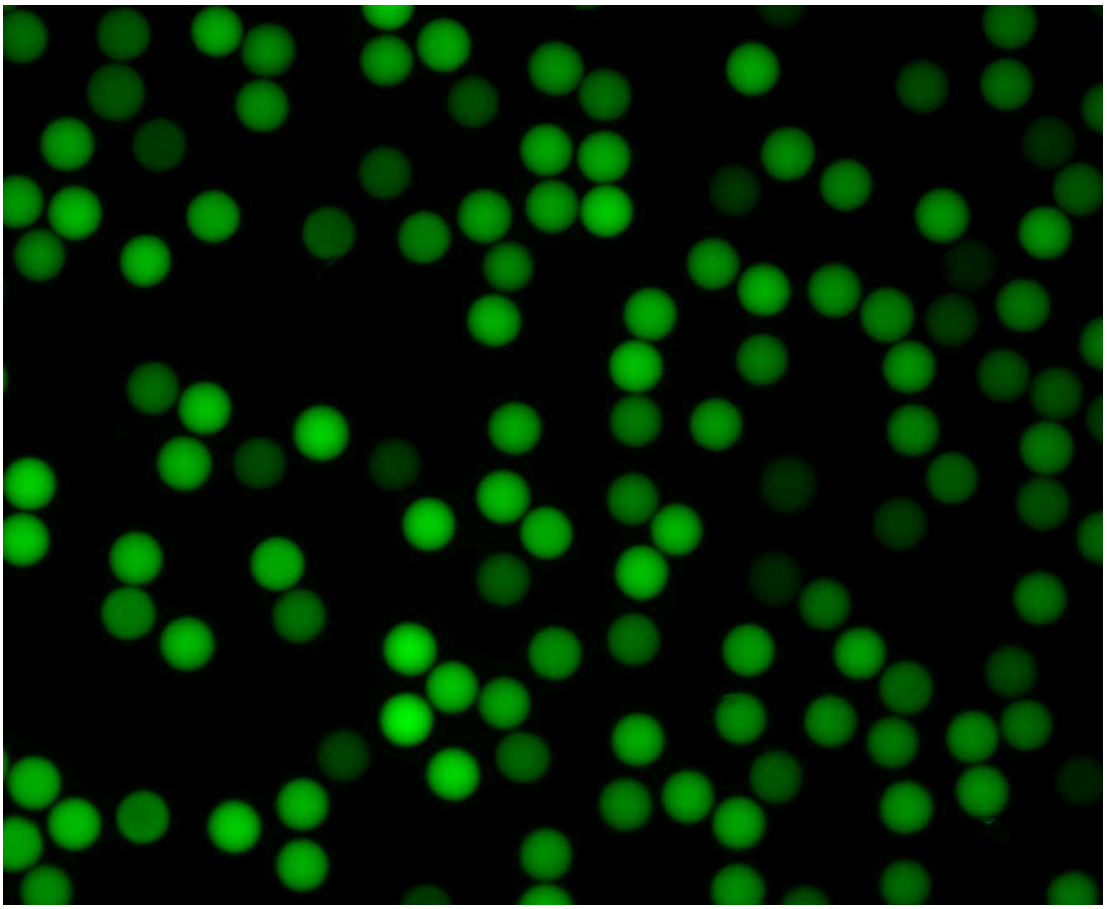
35 Mix by aspiration.

36 Incubate at room temp for 20 minutes in the dark

37 Wash with QC buffer three times (add 1 mL QC buffer, vortex, centrifuge at 1000g 1 min and remove supernatant. At end of last wash, remove supernatant, leave ~10-20 uL total volume). Aspirate final volume to mix.

38 Take 10 uL of each condition, load on a C-Chip device and visualize with fluorescence microscopy with the FITC filter at 10x objective lens. Get around a couple hundred beads imaged for all conditions.

39 Results should be similar to the following:



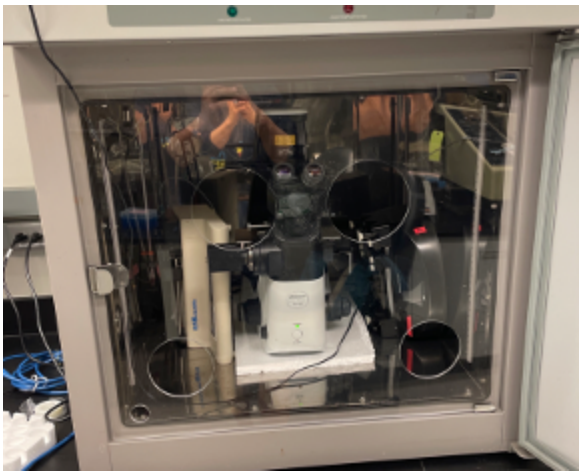
515f_FAM probe on barcoded hydrogel beads. There will be some variation across the beads due, but there should be a very distinguishable signal from the background.

If PE1_FAM shows signal but 515f does not, the extension was unsuccessful. If extension was successful, continue.

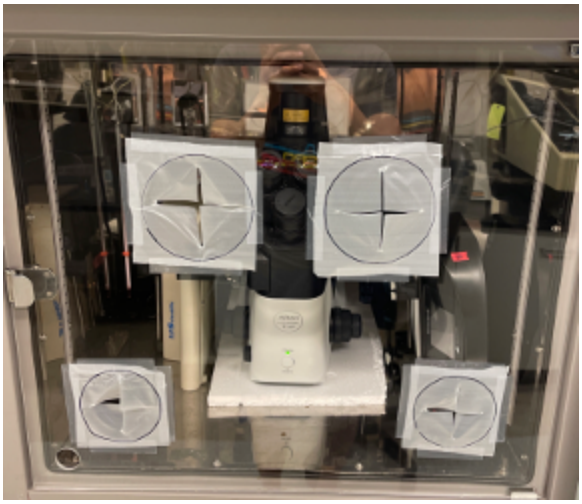
Droplet co-cultivation in microdroplets

40 **Prepare environment for agarose droplet generation.**

All steps for agarose bead generation should be done in a 37 °C environmental controlled room or in a modified incubator. In our cases, we used a modified large oven incubator (VWR Scientific 1535) to maintain a temperature between 37-40°C to keep low-melting agarose suspensions fluid. To limit heat loss, we removed the oven incubator's outer door and replaced the inner glass door with an equivalent-sized, clear acrylic sheet (Optix, 30"x36"x.22") with holes cut by CNC milling to allow operators to adjust the microfluidic device and tubing as well as operate syringe pumps.



Inverted microscope and syringe pumps inside the modified oven incubator. The acrylic sheet to allow user hands to operate equipment replaces the door.



Plastic sheets with "X" cuts are taped onto the holes to prevent excess heat loss. There are 5 holes: 4 for handling equipment, and one (bottom left) for running cords through.

An alternative would be to use an environment-controlled room with the temperature set to 37 C. This would probably be the easiest option if available.

Alternatively, you could use low-melting agarose which remains fluid at room temperature. However, we have not tested this ourselves, and mechanical properties of the resulting gels may be slightly different.

40.1 In the oven incubator, put in two syringe pumps. Run cords through holes so the door can be fully closed.

Attach the microscope camera to the side port of the microscope and attach the microscope connector cable (connected to a computer to run viewing software) to the microscope through a hole. Open the program on the computer to run viewing software.

40.2 Calculate how much droplet oil you need by the amount of samples and volume of droplets you want for each. (Oil flow is at 10 uL/min and aqueous flow is at 5 uL/min). Place a syringe holding the total volume of 2% surfactant (Biorad Technologies, 008-FluoroSurfactant-1G) in HFE7500 Novec oil in one of the syringe pumps. Attach 1 foot of PFTE tubing (Cole-Parmer, 0.022" ID x 0.042" OD) to the end of the syringe and prime the pump so the oil is near the end of the tubing.

40.3 Place the droplet generation device (described in the paper) on microscope stage and turn on the microscope light. Place a short PFTE tubing (4-6 inches) on the outlet of the device. Attach the tubing connecting the oil syringe to the oil inlet.

Adjust the microscope stage and focus knobs to focus on the intersection of the co-flow channels of the microfluidic device.

40.4 Place 1 mL syringes and syringe needles in the incubator oven so they remain warm when working with agarose solution.

41 **Prepare 2% agarose suspension.**

Final concentration of agarose is 1%, make a 2% stock and dilute 1:1 with cell suspension to get to 1%. If you would like to use a different concentration, account for that in the following steps.

41.1 Turn on a heated water bath to 80 °C. Let it heat up during preparation.

41.2 Measure out around 100 mg of low-gelling temperature agarose (Sigma-Aldrich, A9414) on weigh paper. If you have less or more, record the weight. Transfer to a clean, sterile 50 mL Falcon tube.

41.3 Add the appropriate media/buffer to agarose for 2% suspension. For 2%, 100 mg corresponds to 5 mL of buffer/media. Using your recorded exact weight, add the calculated amount of media/buffer to the Falcon tube with the agarose.

41.4 Parafilm the lid of the Falcon tube, so water can't enter. Sonicate the Falcon tube for 5 minutes. Alternatively, just vortex well, but sonicating allows you to keep the suspension at the bottom of the tube.

41.5 Place the Falcon tube upright in the heated water bath and let agarose dissolve for 15-30 minutes.

41.6 Once fully dissolved, filter the 2% agarose solution through a sterile 0.45 um syringe filter (PDVF) into a new 50 mL Falcon tube.

Filtering removes any small particles or agarose that hasn't melted.

41.7 Place agarose suspension in heated water bath upright until you need it.

42 **Prepare cell suspensions**

Cell suspension will be prepared 2X concentrated to be added 1:1 with agarose suspension to obtain final concentration.

For your environmental sample, prepare according to standard procedures to obtain debris-free cell suspensions. Alternatively, if working with cell cultures, generate cultures in appropriate cultivation environments.

42.1 Take 1 mL for each cell suspension and place into clean 1.5 mL microcentrifuge tube and centrifuge at 4000 x g, 00:05:00 . Remove supernatant with pipette tip and refill with 1 mL of your respective media, vortex well. Repeat for 3 total washes.

For last wash, if you would like to concentrate your cells to increase the cell density, add less than 1 mL of PBS or respective media. Vortex well.

42.2 Count the cellular suspension with a disposable haemocytometer (NYC, C-Chip, Improved Neubauer). Dilute to an appropriate dilution and pipette 10 uL into a C-Chip device.

You can use this spreadsheet to determine the cell suspension necessary from cell counts:
Haemocytomer_counts.xlsx
If following the protocol, droplets will be 80 µm diameter.

Tips for counting:

- For an overnight *E. coli* K12 culture in LB, 1:100 (10 uL washed cell culture in 990 uL PBS or media) seems to be good for counting, but will really depend on your cell culture. Can always start with 1:100, but if you need more or less concentrated, can always remake dilutions.
- Wait 10 minutes for the cells to settle more on the bottom of the haemocytometer, this may help in cell counting if most cells are in the same plane.
- Count the total number of cells in 3-5 200 um x 200 um grids.
  The C-Chip is 100 um deep, so there will be multiple planes to scan in visualization. In counting bacterial cells, I usually oscillate between the full range of planes with the fine focus adjustment knob to make sure I count all cells in that grid.

For determining the lambda (average cells/droplet), consider what your biological questions are and that there will be quite a degree of variation based on Poisson statistics. It may also be an option to try multiple lambdas for the same sample.

42.3 Make your 2x cellular suspension according to the calculations.

42.4 In the biohood, combine the 2% agarose suspension, 2x cell suspension, and remaining media/buffer according to your calculations and mix/vortex well. Work fast because if left too

long the agarose suspension will gel. Quickly place back the 2% agarose suspension in the water bath to keep hot.

43 **Generate agarose droplets**

43.1 With 1 mL syringe and 23 gauge syringe needle from the incubator oven, take up the prepared cell suspension. Fill the rest of the syringe volume with air.

To remove air bubbles caught at the plunger, hold the syringe upwards, and quickly jiggle the plunger up and down without losing any liquid. Bubbles will float upwards and pop. Leave the agarose/cell suspension in the plastic syringe, change out the syringe needle with a new one and attach a foot of PFTE tubing to the end.

43.2 Keeping all the suspension in the plastic syringe and not in the needle, move to a syringe pump in the oven incubator, insert the other end of the PFTE tubing to the device inlet, and let everything equilibrate to temperature for a couple of minutes.

If you do not equilibrate to temperature, agarose suspension will gel on the sides of tubing and impede efficient flow. If you find agarose prematurely gelling during droplet generation, keep syringes and syringe needles in an incubator at 50 C or use a heat gun to heat pipette tips or syringe/syringe needles to prevent gelling upon contact with agarose.

43.3 Set the syringe pump to the correct flow rates and settings:

Oil: Set correct syringe settings (Brand, syringe volume) and flow rate: 10 uL/min
Aqueous suspension: Set correct syringe settings (brand, syringe volume, correct side), flow rate: 5 uL/min

Prime the aqueous suspension pump so the liquid/air interface in is right at the entrance of the device. You can either do this manually or by running the syringe pump at 100 µL/min. This might be difficult to see in the oven incubator, so feel free to go slower.

43.4 Place a microcentrifuge tube at the outlet tubing to collect waste droplets and run both syringe pumps. Watch the microscope live video to make sure flows stabilize and droplets are stable.

43.5 Once the initial flow through and non-stable flow droplets have passed through the outlet tubing (wait 1-2 minutes) and the droplet generation is stable, place a new labelled microcentrifuge tube at the outlet tubing.

43.6 Run until enough droplets have been collected. PFTE tubing my pop out of the device or flow becomes unstable due to channel clogging, so stay nearby to monitor.

43.7 If done, close the collection tube and leave in the incubator. If continuing with another sample, dispose outlet tubing and the aqueous suspension syringe and tubing. Repeat for another sample and use a different device.

I find that reusing a device is bound to lead to clogging, so if your sample is limited and sensitive, I would not do that.

44 If incubating droplets, pipette 100 uL of 37 C incubated mineral oil on top of the droplets. Make sure droplets are fully under the mineral oil. If not, add more mineral oil and remove excess mineral oil with pipette tip later. Incubate droplets at whatever condition appropriate, preferably at 37 C or higher.

Do not let droplets sit at room temperature for too long or they will set!

45 After incubator, take droplet tube and let sit for 30 minutes On ice .

Cell lysis of microgels

46 **Recover agarose beads from emulsion**

46.1 Remove as much mineral oil on top of the emulsion with a 200 uL pipette tip without removing too many droplets

- Not all can be removed and some will stick to the sides
- I recommend removing the mineral oil by placing the pipette tip close to one side of the microcentrifuge tube

46.2 Remove as much oil phase on the bottom as possible with a gel loading pipette tip.

46.3 Add 500 uL of PBS-wash buffer and 500 uL of 20% perfluorooctanol in HFE7500 oil (0.22 filtered), vortex well, and centrifuge for 1 min at 1000 g

46.4 Remove oil phase on bottom (1000 um tip is fine). If there is still persistent droplet emulsion, repeat step 3 again, but typically you shouldn't need to. Remove any residual oil with gel loading pipette tip.

46.5 Add 500 uL of 1% Span-80 in hexane, vortex well, and
1000 x g, Room temperature, 00:01:00

46.6 Remove clear hexane layer on top. A dense frothy hexane/water emulsion will be under that, but that doesn't have to be removed.

46.7 Add 500 uL of PBS-wash, vortex well, centrifuge (same settings), and take off the top 500 uL. Skim off the frothy/milky layer on top.

46.8 Repeat previous step 3 times until the liquid is clear, and the microgels are packed well at the bottom.

46.9 Remove most of the supernatant without removing gels, add 500 uL of PBS-wash, aspirate a couple of times to mix, and transfer the suspension to a pluriSelect microcentrifuge filter (70 um) in a clean 1.5 mL microcentrifuge tube (not autoclaved, “nuclease-free” bag)

- The filter can get overloaded, so keep max at 200 uL agarose gels

46.10 Filter by centrifugation

- Try first at 200 x g, Room temperature, 00:01:00 . While a substantial portion will be left on the filter, a substantial portion will be pelleted at the bottom of the microcentrifuge tube.
- If not, resuspend the gels that went through with a 1 mL pipette tip and reload the filter and mix by pipetting up and down to resuspend the gels. Centrifuge again at 300 x g, Room temperature, 00:01:00 .
- If most of the gels are stuck on the filter still, the gels may be too big. You can try upping the g-forces, but you also risk getting gels that are too big through.

46.11 Wash the gels with 1000 uL 10 mM Tris-HCl, vortex, centrifuge 1000 x g, Room temperature, 00:01:00

46.12 Repeat previous step two more times. Remove as much liquid as possible without removing gel pellet.

47 Make 2X lysis buffer (or if there is enough frozen leftover, defrost on ice). The following amount is good for 9 samples. Scale-up as needed.

- 4.470 mL molecular grade H2O
- 100 uL of 1 M DTT
- 100 uL of 1 M Tris-HCl pH 8.0
- 50 uL of 0.5 M EDTA
- 200 uL of 5 M NaCl
- 80 uL of (25 kU/uL) ready-lyse lysozyme

Keep on ice; any unused volume, aliquot in 1.5 mL tubes, store in -20 C

48 Add 500 uL of 2x lysis buffer to the gel suspension. Add molecular grade water to the 1000 uL line on the microcentrifuge tube. Invert a couple times to mix and vortex.

49 Place in 37 C orbital incubator in Styrofoam Falcon tube rack overnight.

50 Set water bath to 50 °C .

50.1 Make 2x digestion buffer (or if there is enough frozen leftover, defrost on ice). The following amount is good for 9 samples. Scale-up as needed.

- 3000 µL of molecular grade H2O
- 300 µL of 1 M Tris-HCl pH 8.0
- 200 µL of 0.5 M EDTA
- 800 µL of 10% Triton X-100 (v/v)
- 500 µL of 10% SDS
- 200 µL of proteinase K (50 ug/uL)
- Keep on ice; any unused volume, aliquot in 1.5 mL tubes, store in -20 °C

50.2 Take tubes with beads from 37 C incubator and centrifuge at 1000 x g, 00:01:00 .
Remove as much supernatant without removing gels.

50.3 Wash with 1000 uL 10 mM Tris-HCl three times, on last wash remove supernatant to 500 uL volume line.

50.4 Add 500 2X digestion buffer, vortex well. Incubate at 50 °C for 30 minutes
Do NOT let sit for longer than 30 minutes.

50.5 While waiting, prepare clean-up buffer. The following amount is for ~4 samples. Scale up as needed. This buffer doesn't keep well (PMSF degrades within a couple hours in aqueous solution) and do NOT ice this buffer (PMSF will precipitate out).

- 4100 uL of molecular-grade H2O
- 50 uL of 1 M Tris-HCl pH 8.0
- 100 uL of 0.5 M EDTA
- 500 uL of 10% Tween-20 (v/v)
- (Add later at step 50.7) 250 uL of 0.1 M PMSF in EtOH

50.6 13. Centrifuge at microgel suspensions at 300 x g, Room temperature, 00:01:00 to remove supernatant.

50.7 Add PMSF to clean-up buffer (as specified in step 50.5), mix buffer by pipetting up and down with 1000 uL tip

50.8 Add 1000 uL clean-up buffer to gel pellet samples, vortex. Let sit at room temperature for 1 minute.

50.9 Centrifuge gel suspension at 300 x g, Room temperature, 00:01:00 and remove as much clean-up buffer as possible without removing gels. If not using today, wash five times in 1000 µL TET buffer and store at 4 °C (leave in 1000 uL TET buffer). If using today, follow droplet barcoding protocol instead.

Droplet barcoding

51 (OPTIONAL) Place sufficient amount of PFTE tubing (3-5 feet), unwrapped 23G syringe needles, microfluidic device, and razor in a glass petri dish. Close dish. Place in Spectroline UV Crosslinker, as close to the lamp with aluminum foil under the dish for 1-2 hour to decontaminate any environmental DNA. Also perform with the bead buffer, agarose suspension buffer, and molecular grade H2O in 50 mL Falcon tubes.

I did this to make sure that any contaminating DNA wouldn't be amplified, as per this article:

CITATION

Woyke T, Sczyrba A, Lee J, Rinke C, Tighe D, Clingenpeel S, Malmstrom R, Stepanauskas R, Cheng JF (2011). Decontamination of MDA reagents for single cell whole genome amplification..

LINK

https://doi.org/10.1371/journal.pone.0026161

52 Determine how much BHB suspension and PCR 1.5x mastermix are needed for the number of samples and
number of actual droplets with both BHB and agarose beads.

When performed correctly, the average BHB encapsulation/droplet is ~0.9, and average agarose microgel encapsulation/droplet is ~0.5

For ~5000 agarose beads barcoded, with appropriate safety factors, it's about 6.5 min, which requires 6.5 uL of packed BHB and 20 uL of 1.3X PCR mastermix. That's too small of a volume to manage, but if you have 5 samples, you multiply that all by 5.

The minimum I would do is 4-5 minutes for each sample, just to get enough volume to work with.
Look at appropriate spreadsheet calculator for details:

barcoding_calculation_estimations.xlsx

53 Setup on an inverted microscope the droplet barcoding device (detailed in the paper). If you have a high-speed microscope camera which can go up to 1000 fps, connect that as well. Place 4 syringe pumps in vertical positions around the microscope.

54 Make concentrated PCR mastermix solution. If you follow this protocol, it should require 1.5x concentrated PCR mastermix:

	A	B	C
	Final concentration (*1.5x)	Component	
	0.2 * 1.5 = 0.3 uM	Pe2_816r (10 uM)	4.5 uL
	1 * 1.5 = 1.5 X	5X SuperFi II Buffer	45 uL
	200 * 1.5 = 300 uM each	10 mM dNTPs	4.5 uL
	1 * 1.5 = 1.5 X	Platinum SuperFi II DNA polymerase	4.5 uL
	1.2% (because of agarose flow also being 1%)	10% Pluronic F-68 (v/v)	18 uL
	0.36 mg/mL (agarose flow also has 0.3)	20 mg/mL BSA	2.7 uL
	Total 150 uL	H2O	70.8 uL

Vortex well and keep on ice until use.

55 Using the 515f-extended BHB suspended in TET buffer in 4 C, spin down whole tube at 1000 x g, Room temperature, 00:01:00 to pellet BHB, add grab the calculated amount of BHB from the packed BHB pellet slowly. Place in a new 1.5 mL microcentrifuge tube. Wash three times in 1000 uL bead buffer. Each time, be careful to not disturb bead pellet and remove supernatant below 250 uL. Move bead pellet and any remaining supernatant into 0.25 mL PCR tubes.

56 For agarose gels suspended in TET buffer in 4 C, spin down whole tube at 300 x g, Room temperature, 00:01:00 to pellet, grab 60 uL from the pellet. Place in a new 1.5 mL microcentrifuge tube. Wash three times in 1000 uL agarose suspension buffer. Each time, be careful to not disturb bead pellet and remove supernatant below.

57 Spin all the PCR tubes with gels and beads on a mini-centrifuge for 20 seconds to pellet. Remove as much supernatant as you can with a 200 or 20 µL pipette tip.

58 Resuspend agarose pellet in agarose suspension buffer to a 30% (v/v) bead suspension. Vortex well.

59 Vortex BHB suspension well (for at least 10 seconds). Cover BHB bead tube in foil to prevent light exposure.

60 To prevent the small volumes prepared from getting lost in the dead volume in the tubing between the syringe and the device, transfer the bead and PCR mix suspensions to the PFTE

tubing, which will be attached to a syringe filled with oil which will displace the volume in the PFTE tubing to flow suspensions into the device.

60.1 Attach a syringe needle to an empty 1 mL syringe, and connect PFTE tubing (~1 ft) of tubing to the syringe needle using tweezers. Push the plunger all the way through until it reaches the 0-mL position.

60.2 Put empty syringe with tubing attached into the syringe pump and set syringe to withdraw at a speed of 25 uL/min. Before starting withdrawal, place the end of the tubing into the suspension and start withdrawing when the tubing is fully immersed. DO NOT introduce air bubbles. Continue until all suspension have been sucked up and allow air to be sucked up for 1-2 cm.

If sucking up bead suspension, a little bead suspension will be left at the end of the tube.

60.3 Take out syringe with the PFTE tubing with the suspension from the syringe pump and cut off the 1-2 cm empty tubing right up to the where the suspension starts.

60.4 Fill a 1 mL syringe with syringe needle with 500 uL of HFE7500, remove air bubbles and connect it to the end of the PTFE tubing holding the suspension. Remove the empty syringe with air from the other end.

60.5 Place the syringe with HFE7500 onto a syringe pump vertically with the needle facing upward. Connect the other end of the tubing to the device. Flow the syringe at 10 uL/min until it reaches the device without actually flowing beads into the device.

60.6 Connect the end of the tubing to the inlets of droplet barcoding device, so that flows are right at the entrance of the device. At the end you will have 3 inlets to the device, one for the BHB suspension, one for the agarose suspension, and one for the PCR mix.

61 Fill a 3 mL syringe with Biorad Droplet Generation Oil (QX200 Droplet Generation Oil for EvaGreen #1864005), connect it to the oil inlet of the droplet barcoding device with PFTE tubing.

This should be a relatively newly bought oil - the stability of droplets in thermocycling decreases dramatically past the expiration date.

62 Connect PFTE outlet tube, flowing into a 0.5 mL PCR tube for collection, but also a 1.5 mL microcentrifuge tube for waste collection.

63 Once everything is connected, switch to 20 uL/min flow for the bead suspension until beads come
close to the device inlet. Once they do, start flowing at 10 uL/min until the beads reach the inlet. For the liquid suspensions, 100 µL/min until they reach the inlet.

64 Remove air bubbles from the rest of the system according to below until air is fully displaced:

- Flow beads at 1 uL/min
- Flow aqueous PCR mastermix at 1 uL/min
- Flow oil flow at 4 uL/min

Continue flowing until BHB and agarose beads become packed.

65 Set the working flow rates for the system until stabilized and then start collecting droplets:

- Flow agarose beads between 0.8-1 µL/min
- Flow BHB between 0.4-0.5 µL/min
- Flow PCR mastermix flow at 3 µL/min
- Flow oil at 5 µL/min

Use high speed camera to check that when dual encapsulation of the beads happens, it is 1:1. If it is not, adjust flows until it is.

66 If flow is good, wait 1-2 minutes for previous droplet flow to exit the outlet tube and into the waste collection tube. Then collect in your collection tube for the appropriate amount of time.

67 Place finished sample on ice under foil. Repeat for another sample, using a different device and agarose bead PFTE tube, but you can reuse the same BHB, PCR mastermix, and oil flow tubing.

68 When finished, remove as much oil from the collection tubes with a gel loading tip. Transfer up to 50 µL of droplets for a 0.2 mL PCR tube for thermocycling.

69 Turn on the UV-lamp (UVP 95-0127-01 Blak-Ray B-100AP High Intensity UV Inspection Lamp with Cool-Touch Housing, 100-watt, 365nm Longwave, 115V) and place it facedown on a safe surface to pre-heat for 10 minutes.

70 UV-treat the generated droplet emulsions On ice under UV-lamp for 10 min

- Make sure the sample is indeed in contact with the ice
- To ensure the sample is cold during UV exposure, I placed the samples in an aluminum foil tray on ice and poured some molecular-grade water in the tray to keep the samples cold).

71 Cover droplet emulsions in each PCR tube with 50 uL mineral oil, making sure the oil covers the droplets.

72 Run thermocycler with the following program:

95 C for 2 min
98 for 30 sec
(98 for 10 sec
60 for 10 sec
72 for 30 sec)*30 cycles

with 2 C/s ramp rates
No lid heating
"Safe" setting or max volume to make sure that the thermocycler heats up the samples long enough.
Will take around 1 hr and 30 min.

73 Remove mineral oil from the top and remove as much carrier oil from the bottom.
Mineral oil may be cloudy after thermocycling so you can let it sit at room temperature for it to clear.

74 Prepare ExoI reagent (1X ExoI buffer with 1 U/uL ExoI) and leave On ice .

44 uL total for 1.1 samples

- 2.2 uL of 20 U/uL ExoI
- 4.4 uL 10X ExoI buffer
- 37.4 uL molecular grade

75 Add 40 uL 1X ExoI buffer with 1 U/uL ExoI to post-thermocycling droplets. Add 30 uL of perfluorooctanol. Briefly centrifuge to merge droplets with ExoI reagent.

76 Incubate at 37 °C for 30 minutes.

77 Perform AMPure XP clean-up of each sample:

- Vortex AMPure XP bead bottle vigorously
- Add 1.5x uL AMPure XP per x uL of sample
- Mix by pipetting up and down, incubate at room temp for 20 minutes
- Spin down briefly with PCR microcentrifuge (not to pellet, just to get any liquid off the cap) and place tube on magnet rack for at least 10 minutes
- Discard unbound liquid, leaving beads alone
- Wash beads with 200 uL of 80% vol/vol FRESH EtOH (made with UV-treated molecular grade water) twice
- Remove EtOH, remove as much residual EtOH as possible with 20 uL tip
- Dry tubes with caps open in the hood for 30 min (cracking is okay)
- Elute in 20 uL of 10 mM Tris HCl 8.0 - mix by pipetting, vortex, spin down to get liquid off the cap
- Incubate at room temperature for 5 min
- Place tubes on magnet to concentrate (2-10 min), transfer the eluate into fresh PCR tube for processing downstream

Library preparation

78 Prepare the following PCR for each of the droplet libraries:

	A	B	C	D
	Component	Stock concentration	Final concentration	Amount to add
	P5_<#> index primer	10 µM	0.2 uM	1 uL

	A	B	C	D
	P7_<#> index primer	10 µM	0.2 uM	1 uL
	NEBNext Q5 HotStart HiFi PCR MasterMix (2X)	2X	1X	25 uL
	10X BSA (20 mg/mL)	20 mg/mL	0.1 mg/mL	0.25 uL
	Nuclease-free H2O			17.75 uL
	Cleaned-up barcoded DNA			5 uL
	Tot			50 uL

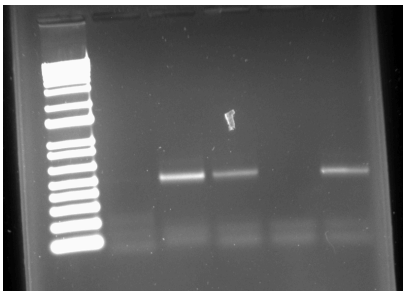
Index primers are specified in the oligo spreadsheet.

79 Run on a thermocycler with the following program:

98 C for 30 s
(98 C for 10 s
68 C for 20 s
65 C for 30 s) x 5 cycles
65 C for 2 min
12 C hold

You may need less or more cycles based on the total initial amount of DNA.

80 Run on a 1.5% agarose gel and stain to check if the correct size. You should get ~500 bp products with very little nonspecific amplification, like below. If you get too much nonspecific amplification, decrease the cycles on the thermocycler.



81 Perform one last AMPure XP bead clean-up.

82 Submit the library for Illumina sequencing following the appropriate protocols.

Citations

Step 51

Woyke T, Sczyrba A, Lee J, Rinke C, Tighe D, Clingenpeel S, Malmstrom R, Stepanauskas R, Cheng JF. Decontamination of MDA reagents for single cell whole genome amplification.
https://doi.org/10.1371/journal.pone.0026161