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HyDrop Bead Generation & PCR Barcoding v1.0 V.8

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ABSTRACT

Protocol for producing dissolvable barcoded hydrogel beads used in HyDrop experiments.

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PROTOCOL CITATION

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Hydrogel bead generation

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Hydrogel bead generation

Here, we will create an emulsion of acrylamide monomers in a carrier oil containing TEMED. The monomer droplets will polymerise and form hydrogel beads. Ideally, you have a bead stock of around 3 mL of beads before you barcode, but 2 mL can work as well. It is best to produce the beads in one single run, from a single bead mixture to prevent disparities in sizes and/or primer concentration within the bead from occurring.

1.1 Prepare bead mix. The volume used here will approximately equate the final volume of dense bead stock that you will produce. The beads will be slightly larger than the droplets generated, but you will also generate small losses during the wash steps.

A	B	C	D	E
Bead mix	**Vol (uL)**	**Stock**	**Final**	
dH2O	920			
Acrylamide	300	40%	6%	
N,N'-Bis(acryloyl)cystamine	220	5%	0.55%	
TBSET	200	100%	10.00%	
Acrydite primer	240	100	12	uM
Ammonium persulfate	120	10%	0.60%	
	2000			

Note that N,N'-Bis(acryloyl)cystamine is dissolved w/v in methanol.

1.2 Prepare TEMED-oil mix by combining TEMED with oil at a 0.4% ratio of TEMED to oil. Note that to encapsulate each volume of bead monomer mix, you will need 800/600 (the ratio between the flow rates) = 1.25 as many volumes of encapsulation oil. In this case, that means you need at least 2.5 mL of oil. To make sure that the oil, which is the cheapest component, does not bottleneck the process, prepare 3 mL of oil (12 uL of TEMED and 2988 uL of oil).

1.3 Transfer the TEMED-oil mix and bead mix mix to 3 mL syringes. Load mix in p1000, insert p1000 tip in the tip of the syringe, withdraw the plunger to suck the prepared mixes into the syringe.
Attach a 25 gauge needle and connect tubing (x cm) to the needles using tweezers

Prime the syringe by **hand**: keep the in a upright position to get rid of air and slowly push the plunger until the air/liquid interface moves all the way to the end of the connective tubing.
Prime the syringe by **pump**: place syringe to pump and flow at a rate of 9500 uL/h until the liquid moves all the way to the end of the connective tubing and you can see it move. This ensures that the syringe is engaged well by the pump.

Connect connective tubing from the outlet port to a 1.5 mL or 2 mL eppendorf tube. Add 300 uL of mineral oil to the collection tube to form a vapour-tight seal. If you forget this, then the fluorinated oil will evaporate during the 65 C baking step after droplet formation, and the droplets will break as a result.

1.4 Use the following flow rates as guidance:

Monomer mix: 600 uL/h
Oil mix: 600 uL/h

Important: the nominal flow rates displayed by your pump should not dictate the final flow rates that you use in your setup. Variations in pump model and microfluidic channel dimensions will lead to different sizes for the nominal flow rates. Due to spin-coating of the wafer, for example, chip features at the outer edge of the wafer will be slightly lower in the z-dimension, resulting in different flow velocities at the same volumetric flow rates. The final size of the bead should be about 50 micrometres in diameter, and this is ultimately what you should tune your flow parameters on. Assuming that your microfluidic channel fully accomodates a 50 micrometre sphere to pass through (no squishing in the channels), you can measure the droplet diameter in the funnel by measuring its circumference (trace a circle and ctrl+m in FIJI) and back-calculating diameter.

The outlet funnel should look like so:

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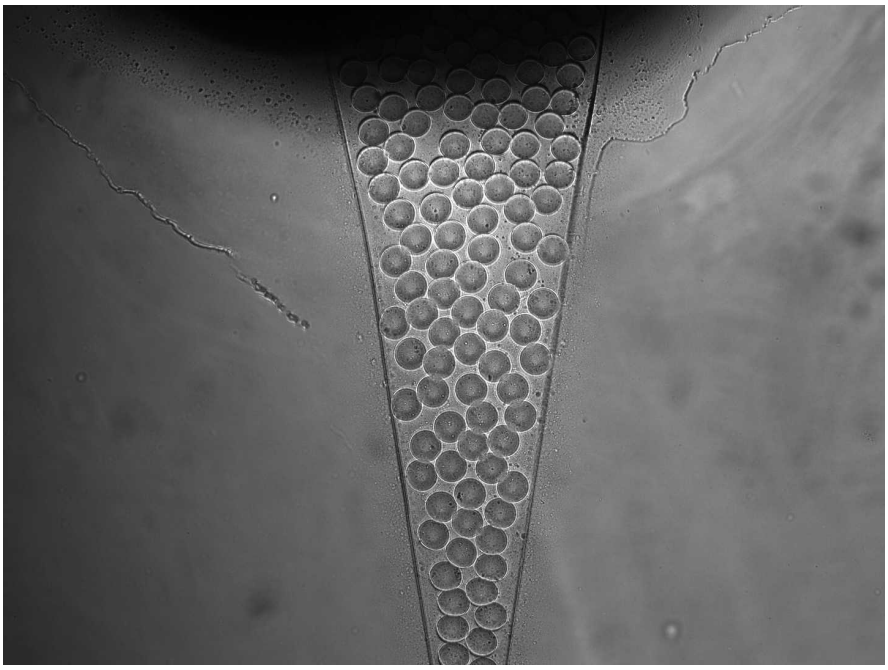
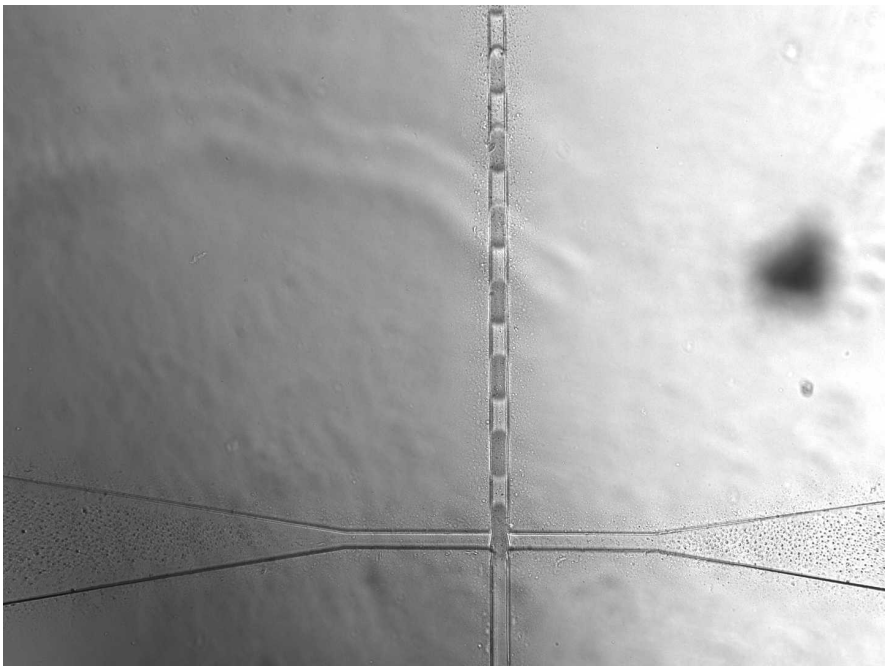


And the flow-focusing point should look like so:



Note that the flow is in a dynamic equilibrium, and that the droplets are formed close to the flow focusing point. If the flow stutters or produces beads of unequal sizes, make sure all tubing is connected properly, and that the flows are not impeded anywhere.

If all goes well, this process will produce beads ~50 um in diameter.
Collect aliquots of 1 mL of beads in a 2 mL eppendorf tube.
Remove excess oil from the bottom and add 400 uL of pure mineral oil to the top to prevent evaporation.

1.5 Double check if layer of mineral oil is present.
Put aliquots on 65C heat block for 14 hours.
Put polymerised beads in 4C fridge until clean-up can begin.

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Buffer mixing

2 Prepare all the buffers below. Most of these buffers are identical to Zilionis 2017, though small changes (such as the PCR buffer) may be present. These buffers may be stored at 4C for at least 3 months. We highly recommend you to filer these solutions using a 0.2 um cell medium strainer.

There is also an excel file below which you can use to manipulate the volumes you want to make or adapt to your stock concentrations (simply change the total volume or stock cell, other cells will adjust accordingly.

20210223_bhb_buffers.xlsx

A	B	C	D	E
1. TBSET - Soln 1010 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	465.85	-	-	
Tris HCl (8.0)	5	1	0.01	M
NaCl	13.7	5	0.137	M
KCL	0.45	3	0.0027	M
EDTA	10	0.5	0.01	M
TX-100	5	10.00%	0.10%	%
	500			

A	B	C	D	E
2. TET - Soln 1020 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	480	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	%
	500			

A	B	C	D	E
3. BWB - Soln 1030 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	490	-	-	
Tris HCl (8.0)	5	1	0.01	M
TW-20	5	10.00%	0.10%	M
	500			

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A	B	C	D	E
4. PCR Buffer - Soln 1040 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	481	-	-	
Tris HCl (8.0)	5	1	0.009999	M
KCl	8.3	3	0.04979502	M
MgCl2	0.75	1	0.00149985	M
TW-20	5	0.1	0.001	
	500.05			

A	B	C	D	E
5. STOP-25 - Soln 1050 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	448.3	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	25	0.5	0.025	M
TW-20	5	10.00%	0.10%	%
KCl	16.7	3	0.1002	M
	500			

A	B	C	D	E
6. STOP-10 - Soln 1060 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	463.3	-	-	
Tris HCl (8.0)	5	1	0.01	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	
KCl	16.7	3	0.1002	M
	500			

A	B	C	D	E
8. Neutralization buffer - Soln 1080 - 500 ml				
Component	**Volume**	**Stock**	**Final**	
Water	425	-	-	
Tris HCl (8.0)	50	1	0.1	M
EDTA	10	0.5	0.01	M
TW-20	5	10.00%	0.10%	%
NaCl	10	5	0.1	M
	500			

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A	B	C	D	E
9. QC buffer				
Component	**Volume**	**Stock**	**Final**	
Water	32.33	-	-	
Tris HCl (8.0)	0.25	1	0.005	
EDTA	0.5	0.5	0.005	
TW-20	0.25	10.00%	0.05%	
KCl	16.67	3	1.0002	
	50			

Barcoded bead synthesis: clean-up

3 Bead clean-up

The beads are now polymerised, but they are suspended in carrier oil. We need to separate the beads from the oil phase by first breaking the emulsion and then washing in degreasing solutions.

3.1 Remove the excess mineral oil from the top and RAN oil from the bottom using a syringe with a tube attachment. This allows you to remove oil without pulling away too many beads.

3.2 Add 1 mL of 20% PFO in HFE, vortex well and centrifuge at 2000 xg and remove the bottom oil phase. Perform this PFO wash for a total of 3 times. The HBs should appear as a solid, packed gel. This is a convenient step to pool the beads: the solid gels can easily be dropped into a common 15 mL tube.

3.3 Per 1 mL of beads, add 1 mL of filtered 1% SPAN-80 in Hexane under a chemical hood. Vortex well, centrifuge at 5000 xg, 30s and remove oil phase. HBs should appear as a packed gel at the bottom of the tube. Remove the top hexane layer. Perform this hexane wash for a total of 2 times.

3.4 Add 10 mL of TBSET and vortex well. Centrifuge 3000 xg, 3 min. and remove supernatant above bead pellet. Be sure to collect all hexane, which appears as a cloudy layer on top of the aqueous phase. Perform this TBSET wash for at least 3 times or further until all hexane is gone.

Barcoding

4 QC

Before proceeding to barcoding, perform acrydite QC according to Zilionis 2017 to check for loss of acrydite primers. You can take a 10x bead sample for intensity reference.

1. Take 10 uL of packed bead stock
2. Wash twice with QC buffer
3. Remove supernatant, leaving about 40 uL left
4. Add 2 uL of 200 uM specific FAM probe
5. Vortex and put on rotator for 30 min at room temperature
6. Wash beads three times with QC buffer
7. Visualise beads under Zeiss Axioplan
8. Settings: 5-FAM filter, 80% light source intensity, 300 ms exposure

You want uniformity in size and intensity. A good QC looks like so:

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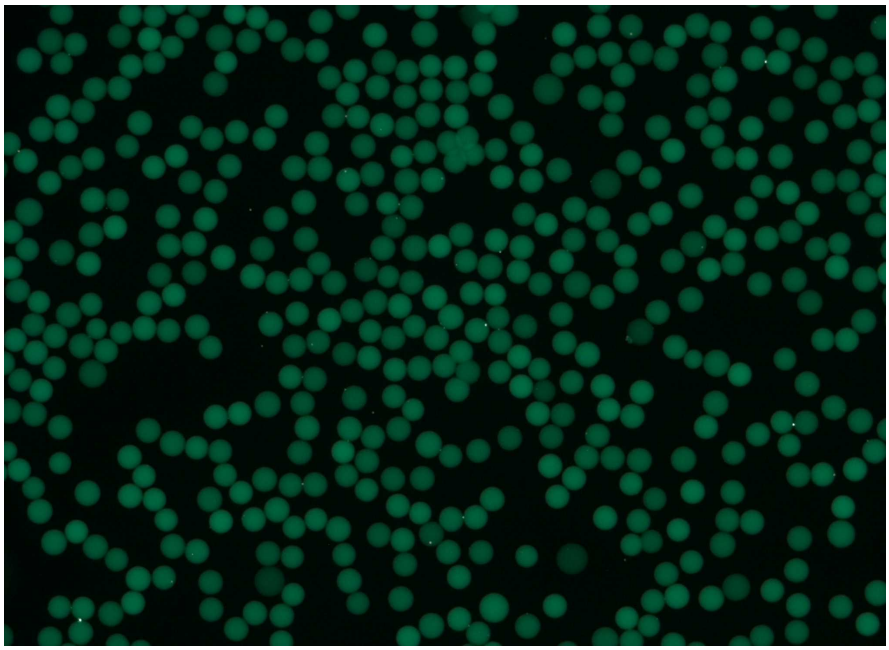

Proceed with the barcoding if QC is satisfactory.

5 Pre-PCR washes

The beads need to be washed several times throughout the barcoding process. A wash entails the following steps:

1. Centrifuge beads at 700 xg, 1 min.
2. Check supernatant for any suspended beads. If beads are visibly suspended, centrifuge again with lower centrifugation speed and braking rate.
3. Remove supernatant with a 10 mL pipette
4. Vortex pellet shortly
5. Add 1 mL of the buffer with which you want to wash
6. Vortex thoroughly
7. Add an additional 9 mL of the buffer
8. Vortex thoroughly
9. Inspect beads for possible clump formation and vortex further if necessary

5.1 Wash the beads twice in Bead Wash Buffer as described above

5.2 Wash the beads twice in PCR buffer as described above

6 Bead split-pooling using Hamilton Liquid Handler

These steps can also be performed manually with an 8- or 12-channel pipette. In that case, distribute all 22.5 uL of beads over a 96 well plate, add 25 uL of KAPA mix to each well, and finally add 2.5 uL of 100 uM unique barcode oligo to each well. You can also pre-mix the KAPA and beads, and dispense 47.5 uL of this mixture instead. Then, perform the PCR program, manually pool the beads and proceed with step 8 of the protool.

We describe the use of the Hamilton robot to distribute beads, KAPA and primers to 96 wells. Please enquire with us if you would like to copy our Hamilton protocol. It is highly likely that the protocol will have to be adapted to your labware and machine specifications.

All 96 well plates must be of the deep well variant and must be placed into a 96 well adapter, otherwise the hamilton coordinates will not match.

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6.1 Open the latest version of protocol in Hamilton control software.
Prepare pipet tips and place in correct position according to protocol
Put full 96 tip rack in column 20, 3rd row counting from the front.
Put 8 tip rack in column 20, 4th row counting from the front.
Place empty working 96 well plate (VWR cat 82006-636) in the first column, 4th row from the front.

6.2 Estimate the volume of beads that you have. Without too many failures, the monomer volumes used above will produce around 2 mL of beads. If you use a volume of 22.5 uL of beads per well, you need 2160 uL total. To account for hamilton pipetting losses, add 20% extra. This means you need 2592 uL of beads. If you don't have enough beads, top off the ~2 mL of packed beads to 2592 uL using PCR wash buffer and dispense this slightly diluted bead stock isntead.

6.3 In the hamilton program, enter the primer, bead and KAPA mix volumes which you will use. For the above volumes:
Beads: 22.5 uL
KAPA: 25 uL
100 uM primer plate: 2.5 uL

KAPA must always be 50%. The final primer concentration here is 2.5 uM, but this may vary in the future.

6.4 Fill column 1 of the bead reservoir plate with the indicated volume of beads.
Place reservoir plate in the first column, 2nd row from the front
Proceed with the program and watch as the machine dispenses 20 uL of beads from the reservoir plate into the working plate.

6.5 Refill the reservoir plate with beads as indicated and continue the program.
Run the last step where the remainders of the beads left in the reservoir plate are distributed over the working plate.

6.6 Fill column 1 of the bead reservoir plate with the indicated volume of KAPA and watch as the hamilton dispenses KAPA in all working plate wells. Refill the KAPA in the bead reservoir plate when prompted.

6.7 Place the primer plate in the correct slot and watch how the hamilton dispenses 5 uL of primer to the working plate.

6.8 Seal the primer and bead plate with BioRad seals.
Spin down the bead plate at 300 xg for 1 minute.

7 PCR

Sub-barcodes are integrated into the hydrogel matrix using PCR.

Shake the plate 30s at 2000 rpm on a room temperature heat block.
Put plate in PCR block and start HB PCR program.

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A	B	C
a. 95C	: 3 mins	
b. 98C	: 20 s	
c. 38C	: 4 mins	
d. 72C	: 2 mins	repeat b-d for 5 cycles
e. 98C	: 1 min	
f. 38C	: 10 min	
g. 72C	: 4 mins	
h. 4C	: Inf	

Meanwhile, set heat block to 38 C
During the 38 C annealing step, take the plate out of the PCR block and shake it for 30s at 2000 rpm on the 38 C heat block. Return the plate and continue. This is crucial, as lack of convection due to the beads will lead to uneven bead barcoding if you don't do this!

8 Post-PCR washes

Now, the hamilton is used again to pool all 96 wells. Then, STOP-25 is added to stop all enzymatic activity. After this, we wash the beads several times to remove free primers. Then, the beads will be incubated and washed in a denaturation solution which removes oligos hybridised to the bead barcodes.

8.1 10m
Return the bead plate to its original position in the hamilton.
Place an empty hamilton reservoir in the correct position.
Place a hamilton reservoir filled with STOP-25 in the correct position.

8.2 20m
Continue with the Hamilton program. The robot will now collect the beads into the reservoir.
Pool the beads into a 50 mL tube. Clean the reservoir with STOP-25 and pool it in the 50 mL tube.

8.3 35m
Keep the 50 mL tube at room temperature for 30 min., flipping it intermittently so that the beads never settle.

8.4 20m
Centrifuge the pooled beads 3 min 800 rcf with braking speed reduced to 3 (to not resuspend beads during the braking in the 50 mL tube).
Remove supernatant and transfer pellet to a 15 mL falcon tube.
Wash the 50 mL tube with STOP-10.
Wash beads in STOP-10 for a total of two times as described above.

8.5 20m
Mix fresh denaturation buffer:

A	B	C	D	E
7a. Denaturation - Soln 1070a - 60 ml				
Component	**Volume**	**Stock**	**Final**	
Water	60.5	-	-	
NaOH	0.938	10	0.150108821	M
Brij-35	1.05	5	0.084016131	M
	62.488			

Resuspend pellet in fresh denaturation buffer, spin down, remove supernatant and resuspend in denaturation buffer.
Incubate with rotation for 10 minutes.
Wash beads three additional times in denaturation buffer, with a 1 minute incubation inbetween every wash. Use 300 rcf centrifugation to prevent bead clumping.

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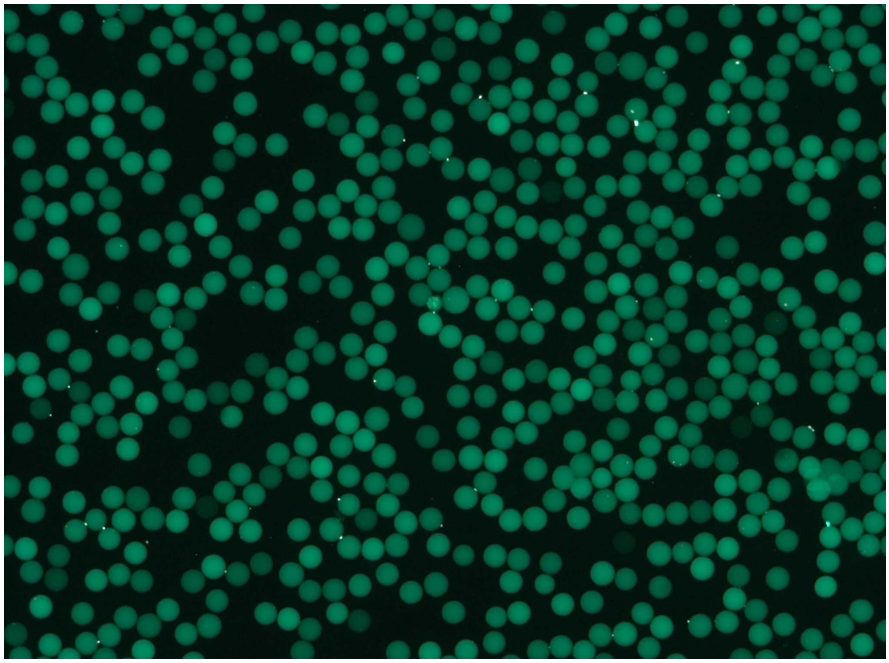
8.6 Wash beads twice in neutralisation buffer. If bead clumping occurs, vortex vigorously and use a P1000 to resuspend the clumps. 15m

8.7 Wash beads twice with TET and filter through a 70 um strainer until no clumps are left. Invert strainer and centrifuge to reduce yield losses. 15m

9 Go back to pre-PCR washes until the barcoding is finished. After the last step, filter beads three successive times to eliminate dust, perform QC as described in Zilionis 2017. 1h 30m

1. Take 10 uL of packed bead stock
2. Wash twice with QC buffer
3. Remove supernatant, leaving about 40 uL left
4. Add 2 uL of 200 uM specific FAM probe
5. Vortex and put on rotator for 30 min at room temperature
6. Wash beads three times with QC buffer
7. Visualise beads under Zeiss Axioplan
8. Settings: 5-FAM filter, 80% light source intensity, 300 ms exposure

A good QC at this stage looks like so:



You want uniformity in size and intensity.

10x beads treated in the same manner look like so:

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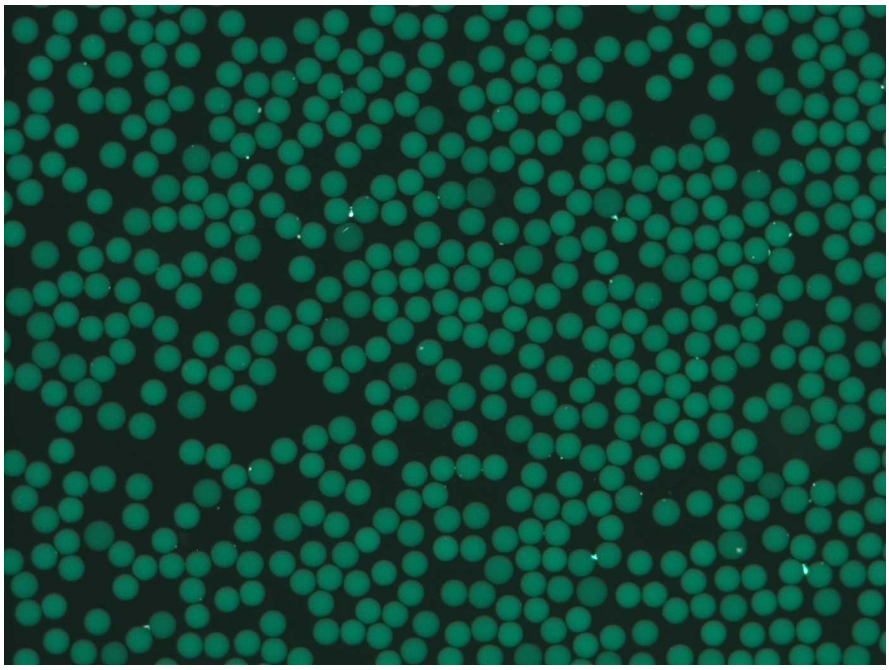

The 10x beads are clearly more uniform in intensity.

Proceed with bead freezing if QC is satisfactory.

Bead freezing 1h 10m

10 Bead freezing

We store the beads in a 30% glycerol freezing/lysis buffer in order to stabilise the weak disulfide bonds. The freezing buffer contains 30% glycerol to prevent freezing artifacts such as changing shape of the beads.

10.1 Prepare bead freezing buffer. This is the same for ATAC and RNA beads. 15m

A	B	C	D	E
BFB	**Volume**	**Stock**	**Final**	**Final drop**
Tris-HCl (pH 7)	3750	1000	125	25
NaCl	900	5000	150	30
MgCl2	300	1000	10	2
Tween-20	1200	100	4	0.80
TX-100	2250	10	0.75	0.15
Glycerol	9000	100	30	6.00
BSA	900	10	0.3	0.06
dH2O	11700	-		
	30000			

Importantly, filter this bead freezing buffer using a 0.2 um strainer to remove any dust.

10.2 Rinse two 15 mL falcon tubes and 3 50 mL falcon tubes with distilled water to remove possible dust particles. This can be skipped if you are confident your plasticware is sterile or dust-free. Keep in mind that a single speck of dust may ruin an entire run. 5m

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10.3 20m
Sequentialy filter the barcoded bead stock using 70 um strainers. To do this, put a strainer on one of your three 50 mL falcon tubes, pour your beads through (collect all remainders using TET buffer). Perform this step a total of three times. If your beads have been freshly barcoded, you can count the two filtrations you performed right after barcoding.

Transfer the final filtrate to one of your clean 15 mL falcon tubes.

10.4 10m
Add 5 mL of BFB to the beads and vortex well.
Spin down at 500 xg for 2 minutes.
Remove and discard the supernatant.
Perform this wash a total of two times.

10.5 5m
Fill the entire 15 mL falcon tube with beed freezing buffer.
Incubate beads in freezing buffer at 4C for at least 3 hours or overnight.

10.6 15m
Take clean/dust-free 8 strip PCR tubes
Centrifuge beads 500 g for 2 minutes, remove supernatant and aliquot packed bead pellet to strip tubes. We recommend volumes between 30 and 60 uL per tube. As a rule of thumb, you need 1/4th the volume of beads per volume of PCR or RT mix during the hydrop experiment. So, 30 uL of beads is good to barcode 120 uL of PCR/RT + nuclei/cell mix - about 2 samples of 2-10k cells each. If you plan on doing large scale experiments, you can store larger aliquots.

Freeze and store beads in -80C.

10.7
When you need the beads, thaw the aliquot on room temperature for a few minutes until all ice disappears and, importantly, remove all supernatant.

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