mini-Drops User Manual

William Stephenson
New York Genome Center
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rev 1.0

Background:

Droplet microfluidics has emerged as a powerful method in genomics enabling the profiling of thousands of single cells. Massively parallel droplet based single cell RNA-seq has been popularized by recent studies including Macosko et al. [Drop-seq], Klein et al. [In-Drop], and commercial offerings such as [10X Genomics]. [Drop-seq] and [In-Drop] were developed in academic laboratories using specialized microfluidic equipment (syringe pumps, microscopes, stirrers, camera etc.) which can be costly. (~$10k) 10X Genomics offers an instrument for single cell RNA-seq however this system is very costly (>>$50k) and is not an 'open' system. This motivated the development of a low-cost control system for a variety of different droplet microfluidic devices. Here we describe the instrument in the context of use for performing [Drop-seq] experiments however one could envision modification to work with other droplet microfluidic protocols.

Further Preliminaries:

If you are planning to use this system for single cell sequencing applications it is important to understand the landscape of technologies and approaches available to researchers. Some approaches may be better suited depending on the researchers budget, resources, desired data or outcome, expertise and the biological samples or systems under consideration as well as the environment in which experiments might need to be carried out. Before embarking on building this project I suggest a full reading of the following papers which represent a snapshot of popular massively parallel single cell transcriptional profiling techniques:

1) Macosko et al. "Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets" 2015, *Cell* 161 1202-1214

2) Klein et al. "Droplet Barcoding for Single-Cell Transcriptomics Applied to Embryonic Stem Cells" 2015, *Cell* 161 1187-1201

3) Zheng et al. "Massively parallel digital transcriptional profiling of single cells" 2016, *Nature Comm.*

4) Gierahn et al. "Seq-Well: portable RNA sequencing of single cells at high throughput". 2017, Nature Methods

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The instrument described is composed of 3D printed parts affixed with electronic and pneumatic components. The instrument replaces the following components of a typical [Drop-seq] setup:

1) Microscope

2) Syringe pumps (3 are required)

3) Magnetic stirrer

4) Microscope camera

The Instrument costs about $550-600 to build this is about 1/2 to 1/4th the cost of single syringe pump which is typically used to generate flow in research-level microfluidic setups.

The instrument uses a Raspberry Pi camera coupled with a laser diode lens to serve as the microscope. The video feed of the experiment is broadcast through a custom written GUI running on a Raspberry Pi 2 model B connected to a touchscreen. Pressure is generated via a micro air pump connected to pressure regulators and PCB mounted micro solenoid valves. The pump and solenoids are controlled through the GUI program which

interface through the PCB to the Raspberry Pi. Pressure is monitored through two PCB mounted pressure sensors and pressure values are displayed to the screen.

Fluid is contained within three cryovials (1.8mL (2) and 5.0mL (1)) which house the 2 aqueous flows (cells and microparticles) and the fluorinated oil respectively. The vials are sealed with custom caps which interface with the output of the solenoid valves (micro air pump) and internal tubing that dips into the fluid within the vial and connecting to the microfluidic chip.

Microparticles are stirred via actuation of a stepper motor (controlled via EasyDriver) that has been affixed with a permanent rare-earth magnet situated on the base of the instrument beneath the fluid vials. When a magnetic stir disc is placed within the microparticle vial the stir disc rotates due to local inversion of the magnetic field via stepper motor shaft rotation.

A typical [Drop-seq] experiment takes about 35-45 minutes for 1mL of each aqueous component. The result is the (Poisson limited) encapsulation of single cells and barcoded microparticles for single-cell RNA-seq. After which the standard [Drop-seq] protocol can be followed to generate single cell libraries.

In addition to the hardware components of the instrument, to perform a [Drop-seq] experiment you will also need:

- Microfludic chip (design mDS_v12.dwg)
- Barcoded microparticles ("Drop-seq beads" Chemgenes Corp. Wilmington, MA)
- Micro tubing PE2 (Scientific Commodities)
- Razor blade or exacto knife
- Nunc round bottom Cryovials 1.8mL & 4.5mL (Thermo Fisher Scientific)
- Magnetic stir disc (V&P Scientific, Inc.)
- Tweezers for tubing placement
- 50 mL conical for collecting droplets
- BioRad QX200 fluorinated oil
- Flexible USB reading light (aids in visualization of the droplets)
- Associated reagents, equipment, pipettes, and tips for downstream molecular biology reactions and sequencing

Before performing an actual Drop-seq experiment it is HIGHLY recommended to perform a number of “dry” runs using toyopearl beads that have not been sent to Chemgenes for synthesis. Using the buffers below, perform a variety of full runs to familiarize yourself with the instrument and the cleanliness requirements to ensure a smooth Drop-seq experiment. Minimize dust/contaminants at all steps of the procedure as these will clog the microfluidic channels.

Procedure:

1) Prepare miniDrops Lysis Buffer and Cell Buffer in addition to the post droplet generation solutions. *Filter all buffers with 0.22-0.45um syringe filters!!*:

miniDrops Lysis Buffer (microparticle buffer)

- Lysis Buffer (makes 10mL): can store large stocks without DTT at room temperature
    - 1.25 mL Ficoll_20% [1.25% final]
    - 100 uL Sarkosyl_20% [0.2% final]
    - 400 uL 0.5M EDTA [20 mM final]
    - 2 mL 1 M TRIS-HCl pH 7.5 [200mM final]

- 50 uL BSA_10% [0.01% final]
- ~6.5 mL molecular H_20
- ** 50 uL/mL 1M DTT [50 mM final]
  - Only add to 1 mL washed-bead aliquots right before experiment

miniDrops Cell Buffer

- BSA: make a 10% solution using BSA powder (Sigma #A8806), store aliquots at -20C
- **PBS-BSA**: make fresh before each experiment (makes 10mL)
  - ~8.7 mL 1X PBS
  - 50 uL BSA_10% [0.01% final]
  - 1.25 mL Ficoll_20% [1.25% final]

The only difference between standard Drop-seq buffers and the miniDrops buffers is that ficoll has been distributed across the two aqueous solutions. This serves to match the viscosity of each buffer. This is required because with the miniDrops a pressure source is used instead of volumetric flow generated via a syringe pump. If fluid viscosities were not matched (by distributing the Ficoll evenly) then the flow rate of cells through the microfluidic chip would not be matched with the flow rate of the microparticles and suboptimal loading of microparticles into droplets would occur.

Droplet Generation Oil

- Bio-Rad QX200 (#186-4006)

Post droplet generation

- 6X SSC
- Perfluorooctanol (PFO) (Sigma #370533)
- TE-SDS
  - 10 mM Tris pH 8.0 + 1 mM EDTA
  - 0.5% SDS
- TE-TW
  - 10 mM Tris pH 8.0 + 1 mM EDTA
  - 0.01% Tween-20

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2) Assemble 1 - 4.5mL cryotube (OIL) and 3 – 1.8mL cryotubes (BEADS, CELLS, WASTE) and 1 – 50mL Falcon tube (DROPS COLLECTION) along with three clean miniDrops custom made vial caps.

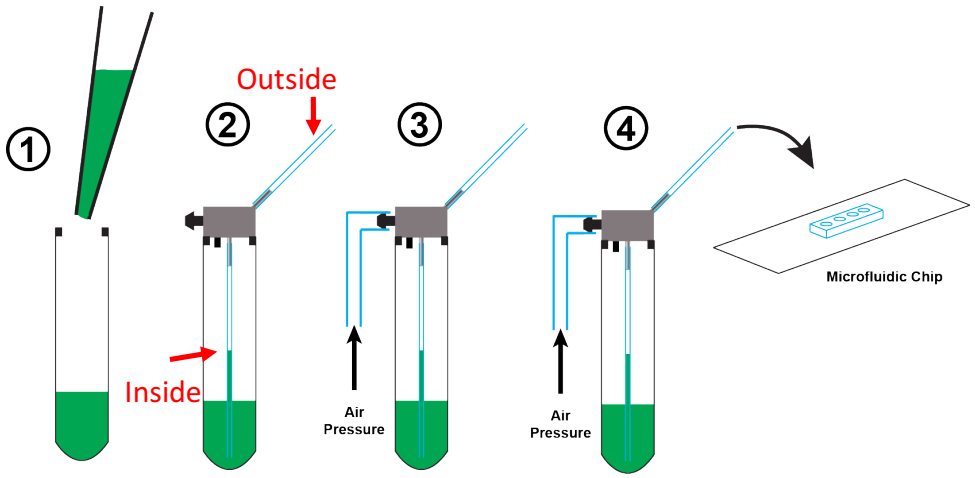
3) Cut and affix PE3 tubing of the appropriate lengths for each respective vial according to the following:
   Aqueous (INSIDE): ~31.5mm (cut 2 of these, one for beads and one for cells)
   Aqueous (OUTSIDE): 67mm (cut 2 of these, one for beads and one for cells)
   Oil (INSIDE): 75mm
   Oil (OUTSIDE): 82mm

Inside refers to the tubing that connects to the stainless steel small tube on the underside of the custom made vial cap and it inside of the vial. This portion of tubing will dip into the solution pipetted into the vial and “sip” from the vial once the head space of the vial is pressurized. It is important to check the length of the tubing as it sits in the vial every time a new tubing is cut. Because each custom made vial cap is different and each person will make slightly different vial caps it is important to use these numbers as a starting point and adjust accordingly. If the tubing is too long it risks being bent and pinched at the bottom of the vial which can impede

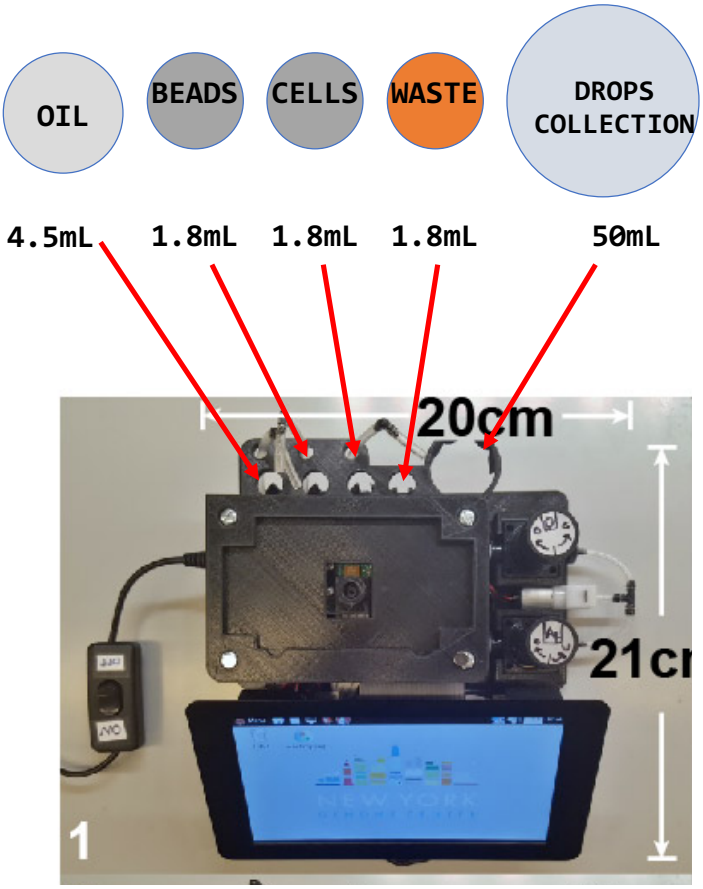
the flow of liquid (cells or beads) through the tubing. Too short and the tubing will only “sip” a small portion of the liquid pipetted into the vial. There is a fine balance of making the tubing just the right length so it permits effective stirring of the disc in the bead vial as well.

Outside refers to the tubing that connects the stainless steel small tube (angled) on the top of the custom made vial cap to the microfluidic chip on the platform of the instrument.

I recommend making a chart with gradations on it for the lengths shown above or your preferred lengths once everything is working. This makes it easy to pull a bit of tubing along the chart and quickly cut it with a clean razor blade. A piece of cardboard with lengths marked with a pen or marker does just fine. After cutting the tubing attach the tubing to the correct stainless steel portion of the vial caps and affix the assembled caps to the respective vials. (See figure below) Remember to add 1 magnetic stir disc to the BEADS vial. (Don’t add the buffers/oil just yet)



4) Assemble the vials into the miniDrops frame according to the following diagram:



5) Double check that you have placed a lens in the correct orientation in the lens housing of the instrument. Place the microfluidic chip on the platform of the miniDrops instrument with the patterned microfluidic designs directly above the window of the platform. Use two small magnets on either side of the microfluidic chip to hold the chip in place. (You should have epoxied two magnets on the underside of the instrument on either side of the window during the build process) Orient the microfluidic chip to observe the junction region of the droplet generation device. Attach the USB reading light to the USB port on the left side of the PCB and turn the light on. Orient the light so that it is illuminating the junction region of the microfluidic chip.

6) Cut a piece of PE3 tubing for the DROPS COLLECTION outlet. This piece of tubing should be sufficiently long (but not too long) to reach the sidewall of the 50mL conical near the bottom of the tube from the microfluidic chip on the platform top. This length will approximately be 150mm.

7) Unscrew the cap to the oil vial and add 4.5mL of BioRad QX-200 OIL to the vial. Carefully replace the cap with the attached tubing.

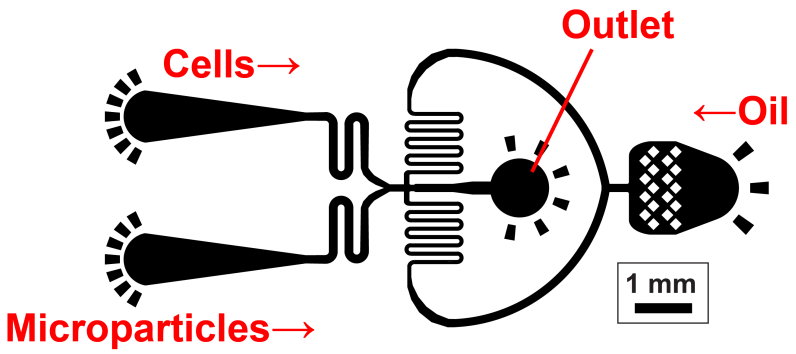
8) Unscrew the cap to the cells vial and add ~1.0 mL of cell suspension in miniDrops cell buffer to the vial. Carefully replace the cap with the attached tubing. (Samples should be at ROOM TEMPERATURE! THIS IS CRUCIAL TO MINIMIZE BUBBLES ENTERING OR FORMING WITHIN THE MICROFLUIDIC CHIP)

9) Unscrew the cap to the beads (microparticle) vial and add ~1.0 mL of Drop-seq beads in the miniDrops lysis buffer to the vial. Carefully replace the cap with attached tubing. (Samples should be at ROOM TEMPERATURE! THIS IS CRUCIAL TO MINIMIZE BUBBLES ENTERING OR FORMING WITHIN THE MICROFLUIDIC CHIP)

10) CONNECT THE PNEUMATIC LINES TO THE CUSTOM CAP EXTERIOR ELBOW FITTING. This ensures that pressure generated from the pump nad through the solenoid valves is delivered to the head space of the vials containing the sample buffers. See the figure-part 3 in step 3) above

11) Immediately after adding the beads to the bead vial, start the miniDrops program by tapping the icon on the touch screen (4 times). This will start the magnetic stirrer automatically and keep the Drop-seq beads in suspension. Verify that the magnetic stir disc is tumbling in the vial through visual inspection by turning the instrument around. If the stir disc is not tumbling the tubing on the inside may be slightly too long. Remove the cap, trim the tubing very slightly and replace the cap to see if the stir disc begins tumbling again.

12) Attach the outside tubings to the correct inlets of the microfluidic chip as seen in the diagram below:



13) Press the Pump ON button on the interface. Adjust the aqueous and oil pressures using the regulators to 0.45PSI and 2.6PSI respectively. Verify all tubing is connected securely and the outlet tubing is oriented to the waste vial.

14) Press the start button (this will actuate the solenoid valves, pressurizing the sample and oil vials thereby pumping fluid through the microfluidic chip)

15) Initially the device will purge air/bubbles through the channels. Adjust the aqueous pressure up to 0.7-0.8PSI momentarily until the beads and cells are visualized in the microchannels and a laminar interface is observed at the droplet generation junction. Once this occurs, decrease the aqueous pressure back down to ~0.45PSI or until droplets are clearly visible through the junction.

16) Adjust the OIL and or the Aq. Pressures so that the outgoing droplets are approximately the width (or just smaller) of the channel containing them. Each of the two laminar flows should contribute approximately 50% to the junction flow.

17) Wait for all bubbles and non-emulsified fluid to transit through the outlet tubing into the waste vial. Once stable droplets have been generated for approximately 30 seconds transfer the outlet tubing from the waste vial to the Drops collection vial. Ensure the outlet tubing is placed against the sidewall of the drops collection vial and not hanging in the middle. (Sometimes this can be achieved by a simple rotation of the vial in the holder) Ensure that a stream of droplets is visible against the sidewall of the 50mL Falcon tube Droplet collection. The droplets should appear as a grainy but consistent width outflow from the outlet tubing.

18) Approximate run time for the instrument is ~40 mins for a 1 mL cell sample. Near the end of the run a high concentration of beads will likely begin to flow through the tubing. Keep an eye on the bead tubing (and the cell tubing) and the bead and cell vials to anticipate the end of the run. Prior to air or final beads running through the microfluidic chip at the end of the run, remove the outlet tubing from the DROPS COLLECTION vial (50mL Falcon tube) and move it to the waste vial. Then shut the solenoids by pressing OFF. If the experiment is over press the OFF button and shut down the raspberry pi as usual. From this point on the protocol follows the Drop-seq protocol exactly.

Troubleshooting:

A) Dirt/hair/beads are clogging the channel:

FILTER ALL BUFFERS!!! Using 0.22-0.45um syringe filters, remove particulates from all buffers prior suspending cells/microparticles. If dirt or hairs are seen to block of clog a portion of any of the microchannels, try tapping the vial which is fluidically connected to it. This may or may not dislodge the foreign particle. If it does not dislodge and or poor droplet formation is a result of the contaminant and the clog persists, transfer the outlet tubing to the waste vial, stop the run by pressing stop, then transfer all tubing connections to an adjacent microfluidic chip, ensuring the correct tubing is placed in the correct port of the adjacent chip. Initiate the experiment again by pressing start and adjusting pressures for optimal droplet generation. Finally, after ~30 secs of stable drop generation transfer the outlet tubing back to the drops collection tube.

B) Screen went DARK:

If the screen goes dark, don't panic, its just the screen saver, lightly touch the leftmost portion of the touchscreen (Where the camera feed is) to wake the screen.

C) The junction flows don't look 50%/50%:

There is probably a clog or a bubble somewhere in the microchannel. Try tapping the vial which is fluidically connected to the channel in which the bubble or clog is observed. Sometimes briefly increasing the aq. pressure can force bubbles through the device. The outlet tubing should be in the waste vial when this is attempted. Adjust the pressure back down for stable droplet generation. Return the outlet tubing to the drops collection after ~30 sec of stable droplet generation. If the clog or bubble persists and is disturbing the laminar interface or interefering with even bead/cell flow into droplets a new chip will need to be used. Stop the solenoids by pressing OFF and transfer all tubing connections to a fresh clean chip. Resume the procedure from step 12 above.

D) Instrument won't turn on:

If the instrument doesn't turn on, first ensure that the correct power supply is being used: 12V, 5A AC-DC converter. Power is then supplied through a barrel jack to the instrument on the left side at the Printed circuit board. Make sure the barrel jack connecter is fully inserted into the receptacle. If the instrument still won't turn on, check the front two power connections on the printed circuit board from the raspberry pi and the touchscreen. Check for the raspberry pi lights to turn on and make sure the proper micro-SD card is inserted into the raspberry pi.

E) Beads are not entering at an even density/distribution/concentration:

If beads are entering the microfluidic chip sporadically, i.e. not at a uniform concentration then look to see that the magnetic stirrer is working and look to the vial and ensure that the stir disc is inverting/tumbling. Recheck proper tubing lengths from the vials to the microfluidic chip. Internal tubing that is too long can prevent the stir disc from tumbling and thus properly stirring the Drop-seq microparticles. Drop-seq microparticles should be washed (2X in miniDrops lysis buffer) thoroughly before use in an experiment.