inDrop™ Single Cell Encapsulation and Reverse Transcription Training Protocol, Version 2.1

Table of Contents

A. Reagents needed for the inDrop™ Single Cell Reverse Transcription Protocol
B. Cell Compatibility
C. Step-by-Step Protocol for Barcoding Cells
   I. Instrument Setup
   II. Silanizing the microchip
   III. Hydrogel Beads, Cell and RT/Lysis Buffer Preps
   IV. Loading Materials in to the Microchip
   V. Initiating and Optimizing Gel Bead Encapsulation
   VI. cDNA Synthesis
   VII. Instrument Shutdown
A. Reagents and Equipment for the inDrop™ Single Cell RT Protocol

What is Provided by 1CellBio

- Wash Buffer
- Naked Hydrogel Microspheres (NHMs) 1.2mL /kit
- Mock Gel Concentration Buffer
- Mock RT Premix
- HFE 7500
- OptiPrep - Density-matching Agent
- Droplet-making Oil
- Demulsifying Agent
- PBS
- 1 mL syringes
- Syringe needles
- 3mL syringe
- Collection tubing
- Microfluidics Chip
- Colored Mineral Oil
- Pipette tip assemblies
- NHM loading assemblies
- Storage Buffer

Reagents and Equipment Needed and not Provided by 1CellBio

- 1.5 mL DNA LoBind tube (Eppendorf AG, Cat. No. 022431021)
- Thermo-conductive tube rack (VWR Scientific Cat. No. 95045-464)
- UV lamp (UVP Model B-100 AP, VWR Scientific Cat. No. 36595-020)
- Heat block for 1.5 mL tubes (VWR Scientific Cat. No. 12621-084)
- Aluminum foil
- Razor blades or scalpel
- 0.22 μm syringe filter for silanization (VWR, Cat. No. 28145-493)
- Centrifuge (Eppendorf AG, Cat. No. 5424R)
- Vortex (Vortex Genie or similar)
- Hemocytometer and cover slips (If training with cells)
- A pair of tweezers

Figure 1. Collection tube of microchip, containing all components to create barcoded cDNA
D. Step-by-Step Protocol for Practicing Barcoding Cells

I. Instrument Setup

1. Log into the laptop provided by 1CellBiO – the login name is “inDrop”

2. Click on the desktop icon labeled “Thor”. That opens the software that controls the instrument and implements the inDrop process.

3. Remove the hydrogel bead syringe assembly from its designated package and mount it on the pump marked “Beads” (Pump 0). Note – the syringe is shipped pre-filled with hydraulic fluid specific to the loading the hydrogel beads.

4. Prime the tubing by using Thor and infusing the hydraulic fluid from the syringe into the tubing. Once a drop forms at the tip of the tubing, stop the pump and remove the excess fluid.

5. Mount the designated droplet making oil syringe on to the pump marked “Oil” (Pump 1).

6. Remove a syringe with attached pipette tip assembly with red-colored mineral oil and mount in the pump marked “Cells” (Pump 2).

7. Place the pipette tip in the holder attached to microscope light source arm with the tip pointed upwards.

8. For Pump2, activate Infuse and set the pump rate = 2000 ul/hr. This will fill the pipette tip with mineral oil. Wipe any excess oil from the end of the pipette tip when it fills with mineral oil.

9. Repeat steps 6-8 for the syringe pump marked “RT/Lysis Mix” (Pump 3).

10. Move to the next step of preparing the hydrogel beads once all syringes are mounted and primed.

II. Silanizing the microchip devices

1. Access the 1CellBiO website (www.1cell-bio.com) and go to the Resources page.

2. Download and follow the “Chip Silanizing” protocol to prepare the microchip device for use.

   Critical: Use low lint tissue to remove excess silanizing fluid.

III. Hydrogel Beads, Oil, Cell and RT/Lysis Buffer Preps
A. Hydrogel Bead Prep
1. Concentrate the barcoded hydrogel microspheres (NHMs) in a 1.5 mL Eppendorf tube by spinning at 5000 g for 2 min. Carefully examine the tube to be sure you can distinguish the bead pellet from the supernatant.

2. Carefully transfer 300 µL of the concentrated NHMs to a new 1.5 mL Eppendorf tube and begin the NHM washes
   Note: 300 µL is sufficient for barcoding 20,000-30,000 cells.

   Critical: minimize the exposure of NHMs to light prior to encapsulation. Ideally all handling will take place in dim lighting.
   Tubes holding the NHMs should be kept away from direct light and covered, e.g., wrapped in foil.

3. Wash the NHMs three times as follows:
   a. Add 1 mL of washing buffer.
   b. Vortex briefly.
   c. Centrifuge at 1,000 g for 1 min.
   d. Remove the supernatant, do this carefully so as not to disturb the bead pellet.
   e. In the last wash, leave ~500 µL in the tube.

4. Add 500 µL of Mock Gel Concentration Buffer, vortex for ~6 seconds, and spin the NHM suspension at 5,000 g for 1 min.

5. Using a p1000 pipette remove as much of the supernatant as possible. 300 µL of close-packed NHMs should now concentrate to around 150-200 µL.

6. Spin down the tube at 5,000 g for 1 min, and use a p200 pipette tip to remove any remaining supernatant.

B. Cell Prep
1. Adjust the concentration of cells to be 100,000 cells/mL, or less, in 1X PBS containing density matching reagent. The cell suspension should have an 18% optiprep to cell suspension.

   Note: As much as possible, keep the cell sample cool on ice while preparing for a run.

   Note: Where possible, prepare more cell phase than you anticipate you will use, ideally a volume of 500-600 µL. When the number of available cells is very low, e.g., a total number of 1,000 cells, use a final volume of at least 100 µL.
C. RT/Lysis Buffer Prep
1. Prepare 200 μL of Mock RT/Lysis Mix.

IV. Loading Materials into the Microchip
A. NHM Loading
1. Compute how many NHMs are needed for the number of cells to be encapsulated and from that estimate the time required to load the NHMs into the delivery tubing based on the equation below.

2. Prime tubing of NHM pump by, on the software, increasing the flow rate to 2000ul/hr. Press “Run” and wait until a droplet of fluid forms at the tip of the tubing.

3. Let the pump run for 10 seconds and press “Stop” on the software

4. Insert the tip of the tubing into the washed NHMs.

5. Set the pump flow rate to 500uL/hr. Make sure the pump is in Withdraw mode.

6. Press “Run” on the software. The NHMs will now be aspirated into the tubing. Time the aspiration and click “Stop” after the specified time has elapsed.

**Critical: Make sure the tip of the tubing stays fully immersed in the NHM pellet. If the tubing tip comes out of the NHM pellet, stop the pump and re-immers the tubing tip into the pellet and resume the NHM aspiration.**

Calculate the Volume of NHMs to be Loaded and Loading Time

**Inputs**
- Number of cells to be barcoded Ncells: User selected
- Bead-to-cell ratio: 13:1

First compute the total number of NHMs, NNHM, required for the experiment:

$$\text{NNHM} = 13 \text{ NHM/cell} \times \text{Ncells}$$

Second, compute the volume of NHMs to be loaded:

$$\text{Volume (uL)} = \frac{\text{NNHM}}{2,100} \text{ NHM}$$
Lastly, compute the time, $T$, needed to aspirate this number of NHMs

$$T \text{ (min)} = \frac{\text{NNHM}}{(300 \text{ NHM/s})/60}$$

**Example**

<table>
<thead>
<tr>
<th>Number of cells</th>
<th>8,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of NHMs</td>
<td>104,000 NHM</td>
</tr>
<tr>
<td>NHM volume</td>
<td>50 ul</td>
</tr>
<tr>
<td>Time to aspirate</td>
<td>6 min</td>
</tr>
</tbody>
</table>

**B. Cell and RT/Lysis Buffer Loading**

1. Once your cell and RT/Lysis mixes are prepared, transfer the Eppendorf tubes to the chiller block.
   a. If you choose not to use the chiller block, transfer the Eppendorf tubes to a small container of ice that will fit on the microscope stage

2. Place the chiller block containing the Eppendorf tubes on the right side of the stage.

3. Set the flow rates of Pump 2 (Cells) to 9000ul/hr and infuse, make sure to there is mineral oil all the way to the bottom of the tip. Wipe the tip off and place it in the cell solution.

4. Repeat step 3 using Pump 3 (RT/Lysis Buffer) and place your tip assembly into your RT/Lysis solution.

5. Set Pump 2 and 3 to ‘Withdraw’ on the software, and make sure the flowrate is set to 2000ul/hr.

6. Run Pump 2 and Pump 3 until the desired amount of cells and buffer are withdrawn into the tip assembly.

**C. Connecting to the Microchip**

1. Set the flow rate of Pump 0 (Hydrogel Beads) to 2000ul/hr and infuse until liquid comes out of the tip. Stop the flow and remove any excess liquid from the end of the tubing.

2. Set the flow rate of Pump 1 (Hydrophobic Oil) to 2000ul/hr and infuse until liquid comes out of the tip. Stop the flow and remove any excess liquid from the end of the tubing.

3. Repeat for the cells and RT/Lysis solution
4. **Gently** insert the tubing and pipette tips into the corresponding inlets of the microchip in the following order:

   a. The pipette tip with cells into the Cell inlet.
   b. The pipette tip with RT/lysis Buffer into the Buffer inlet.
   c. The tubing with NHMs in the NHM inlet.
   d. The tubing with the hydrophobic oil in the Oil inlet.
   e. A collection tube in the microchip outlet to transfer the emulsion to an Eppendorf tube.

**Critical:** Do not press the tip assemblies all the way into the inlet of the PDMS, if pushed too hard, the tip will contact the glass slide and disrupt the flow.

![Fluidic microdevice layout](image1)

*Figure 2: Fluidic microdevice layout*

![A fully connected microchip](image2)

*Figure 3: A fully connected microchip*
V. Initiating and Optimizing Gel Bead Encapsulation

1. Start by clicking “Prime Chip” in the software window. Prime Chip starts the flow of fluid into the microchip to push out the air bubbles in preparation for starting an experiment. Table 2 below details the flow rates to use in each phase of device operation. **Note:** these flow rates are preset in the Thor software.

2. Once beads are flowing into the bead inlet closely packed as shown in Figure 5, click on “Run Experiment”. This will adjust the flow rates to preset values that can be overridden and changed by the user.

![Image of Thor software](Figure 4. Image of Thor software)
3. Adjust the flow rate of NHMs to reach 85-95% occupancy. It may be necessary to fine tune the flow rate in increments of 5 μL/h.
   a. The occupancy rate is measured by taking a video of the drops downstream from the drop forming region. Counting the drops and hydrogel beads per drop determines the gel bead encapsulation percentage.

4. Once flow rates are stabilized allow the system to run for 1-2 min until the emulsions produced initially are displaced from the outlet tubing.

Table 2: Microdevice Flow Rate Conditions

<table>
<thead>
<tr>
<th>Prime Chip (μL/h)</th>
<th>NHM Phase (Pump 0)</th>
<th>Droplet-Making Oil (Pump 1)</th>
<th>Cell Phase (Pump 2)</th>
<th>RT Premix (Pump 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run Experiment (μL/h)</td>
<td>100</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>40</td>
<td>360</td>
<td>250</td>
<td>250</td>
<td></td>
</tr>
</tbody>
</table>

5. Collect the emulsion into a 1.5 mL Eppendorf tube placed in a chilled thermos-conductive rack. Add 200 μL of mineral oil to a 1.5 mL tube and insert the collection tube below the mineral oil.

*Critical:* Mineral oil is necessary to prevent droplet coalescence due to evaporation.

*Critical:* Keep the emulsion cold by using the chiller block.
6. Monitor the gel bead-cell encapsulation with the high speed camera. Adjust flow rates, if necessary, to maintain optimal operating conditions.
   a. There should be at most 1 NHM in each droplet and about 90% of all droplets should contain beads. Avoid having two NHMs per droplet. Adjust the flow rate of NHMs in 5 μL/h increments to ensure that these conditions are maintained. NHM occupancy can be determined from short movies recorded at the outlet of the microfluidic device or simply monitoring the droplet flow using the Strobing Mode or pausing feature in the software. The Strobing Mode will show the flow conditions at 2 fps.

b. Ensure that the cell encapsulation rate remains constant by monitoring the cell inlet. To confirm cell count, record a ~10 sec long movie, count the number of cells flowing through, and calculate the cell count per second. The Standard Mode in the software will record the movies at 150 fps. When you play a saved movie, check the total frames of that movie. To determine the size of that movie, divide the total frames by 150 fps.

Figure 6. Layout of Thor software identifying key features

c. Ensure that the droplet size and formation rate remain constant. Confirm the droplet size. Droplet volume in picoliters (pL) is given by

\[ V = \frac{(F \times t)}{(c \times N)} \]
where \( F \) is the sum flow rates (\( \mu \text{L/h} \)) of all aqueous phases (e.g. hydrogel beads, cell mix, and RT/lysis mix), \( t \) is the time (sec) to count \( N \) droplets, and \( c \) is a factor to correct for unit differences (\( c = 3.6 \text{ sec } \mu \text{L /pL*h} \)).

d. By using the flow rates indicated in the Table above, the droplet size should 3.0-3.5 nL and can be adjusted by changing the flow rate of the droplet-making oil.

e. To verify few droplets contain multiple cells either by estimating the number of cells per droplet using the cell flow rate, the cell density, and the droplet volume, or collect a sample of droplets and estimate their occupancy on the instrument using a hemocytometer.

f. To calculate the time of encapsulation required to collect the desired number of cells, you can use the “Calculator” on the right top corner of the software. When you click on the calculator button, a new box will open and ask you to enter the number of cells you want to collect, the number of cells you counted, the length of the movie (seconds) you saved. By entering this information, the program will calculate the time required to collect the sample. Press “Start” to commence the encapsulation counter.

7. Once the desired number of cells is encapsulated, unplug the tubing from the outlet, stop the pumps, and let the emulsion in the tubing drain into the collection tube by gravity.
VII. Instrument Shutdown

1. Flush the black NHM tubing with the storage buffer by running the pump at 2,000ul/hr for ~1 min. It is recommended to flush the tubing into a Lobind Eppendorf tube. The tube can be treated as a recovery tube for the NHMs. Once complete with the flush, place the sealing cap on the tip of the tubing.

2. Unplug the oil tubing and insert the sealing cap in the end of the tubing. Park the tube in one of the tubing holders on the microscope.

*Figure 7. Set up for collecting sample*
3. For both the Cell and RT/Lysis Buffer pipette tips, set the flow rate to 9,000 ul/hr and click “Withdraw” and “Run” to withdraw the colored mineral oil into the tubing. As soon as the meniscus of the colored mineral oil is visible in the tubing, click “Stop”.

4. Remove and discard the used pipette tips.

5. Insert the end caps into each tubing and place the tubing in one of the tubing holders on the microscope.