*2016-6-28*

**"Pre-flight checks" for inDrops single cell transcriptomics**

Preparing good cell suspensions is the most difficult part of single cell transcriptomics. Expect at least 1-2 attempts to get good quality material before an inDrops run.

Overview of requirements:

1. Plan experiment: decide on number of samples, number of cells per sample, and plan just-in-time cell preps.
2. Consider the availability of cells from your sample
3. Know the % of your cells is the population of interest
4. Produce high-quality single cell suspensions
5. Confirm cell concentrations
6. Confirm cell suspension buffer doesn't inhibit RT (if not using 1X PBS)
7. Confirm cells viable between dissociation and encapsulation
8. Control for free-floating mRNA in sample (particularly for primary tissues)

*Plan the experiment: decide on number of samples, number of cells per sample, and plan just-in-time cell preps*

inDrops can process ~10k cells/hour, but for many experiments it is sufficient to collect just 3-5k cells per sample, and multiple samples may be desirable (biological replicates, time series, dose-response, control/perturbation, etc). Plan on providing 2-3-fold more cells than will be encapsulated, in order to have backups in case of unforeseen problems with library prep, and for use as technical replicates.

Minimum ½ hour per sample – realistic 6 samples per day – 8 samples possible. System prep takes 1 hour. Samples require 2h of processing time after they are inDroped to RT the mRNA.

Consider your sequencing budget: current users are often sequencing 9,000 cells with 450 million reads on a NextSeq, which runs roughly $1400-$2200 (unless you wish to carry out very shallow sequencing). This may set the scale of your initial experiments. While lower depth of coverage may be possible, initial experiments usually require higher depth.

Plan "just-in-time" experiments: viability of many primary and cultured cells begins to drop after more than 1 hour on ice. It is highly desirable for cells to be processed immediately from tissue or culture. If possible, plan to do the final dissociation/clean-up at the TC room next close the inDrops system. If this is not possible, minimize the time your cells will sit on ice before processing. If FACS is required, plan to go directly from FACS to inDrops with no delay. If you are preparing multiple large samples, consider sending each sample for inDrops as soon as they are ready.

*Consider the availability of cells from your sample*

inDrops can process very rare samples, e.g. just a few thousand cells in total. It is much easier to work with more abundant samples. If possible, design your experiment to provide at least 25,000 cells per sample. This will reduce risk of losing the sample due to technical errors, and makes sample handling much simpler. While only 3-5k cells will be encapsulated it is easier to handle larger volumes during process.

*Produce high-quality single cell suspensions*

It is critical to have fully dissociated cells, because we cannot exclude doublets or clumps. Dissociation for single cell analysis is generally more stringent than required for normal cell culture. But over-dissociation can kill cells. Passing cells through a 10/15/20/40µm strainer after initial dissociation can get rid of most doublet/clumps (choose strainer size according to cell type).

Cell suspensions should always be inspected manually on a hemocytometer, using Trypan Blue to assess viability. (They can be scored using an automated cell counter but only in addition to the manual inspection). Count doublets/clumps as single events. Count % events that are not single cells (ideally < 5%). Dead cell fraction should be low.

A typical dissociation protocol is 5 min Trypsin-EDTA @ 37°C with multiple pipetting up/down to break clumps. Then neutralize Trypsin and pass cells through 40µm strainer. If needed pass again through 20µm strainer. Primary tissues may require more complex dissociation methods.

*Confirm cell concentrations*

To avoid pelleting cells, cells can be kept after dissociation in media/serum at a concentration of 800k/mL. (The cells will be diluted 10-fold before RT, so carry-over media/serum will not inhibit RT).

Alternatively, if there are few cells then cells can be pelleted and resuspended in 1XPBS at a final concentration of 200-300k/mL. The total volume can be as low as 50µL. (For lower volumes, please consult with us).

If you need to prepare your cells at lower concentration, please consult with us.

*Confirm cell suspension buffer doesn't inhibit RT (if not using 1X PBS)*

If you require maintaining your cells in any buffer other than PBS, you must ensure that it does not inhibit the RT reaction. (E.g. the buffer should not contain Calcium). You can consult us on known buffers. You can test your buffer by adding it to a control RT reaction on purified RNA and measuring yield of a housekeeping gene by qPCR.

*Keep cells viable between dissociation and encapsulation*

The inDrops experiment takes 30-60 minutes but cells might end up sitting on ice for longer. To test if they will survive, after dissociation into the final buffer, keep cells on ice for 1 hour, then mix/resuspend the cells by pipetting. Inspect the cell viability on a hemocytometer with Trypan blue.

If cell viability is low, repeat for 30 minutes and see if they are viable. (We can try to collect all cells within 30 minutes if needed). Also, try adding 1%w/v BSA to cells, or keeping them at high concentration with 10% serum.

While dead cells can be sorted based on their transcription profile in the final sequencing, this will waste reads and dead cells also leak RNA into the solution and end up creating high background noise for the whole experiment.

*Control for free-floating mRNA in sample (particularly for primary tissues)*

This test is optional but highly recommended.

After generating the final cell suspension (using exact conditions of real experiment), dilute cells in cold 1X PBS to a final concentration of 80k cells/mL.

Transfer 10µL of the cell suspension into a PCR tube (tube 1). Pellet the cells by centrifugation. Transfer 10µL of the supernatant into PCR tube 2. Take 10µL of clean 1XPBS into PCR tube 3.

Carry out RT-qPCR against a house-keeping gene for tubes 1-3 to assess the amount of free-floating mRNA in tube 2, compared to tube 1. Tube 3 is a negative control. Add 0.5% Igepal CA-630 to the RT reaction mix in all tubes to lyse the cells prior to RT.

If free-floating mRNA levels are high, optimize cell dissociation/wash steps until levels are at least 100-fold lower than in cells.