2016-9-23

“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. In case of problems you may require multiple attempts.

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. Produce high-quality viable single cell suspensions
4. Confirm cell concentrations
5. Confirm cell suspension buffer doesn’t inhibit RT (if not using 1X PBS)
6. Confirm cells viable between dissociation and encapsulation
7. 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 most experiments it is sufficient to collect just 3-5k cells per sample.). Unless you have a very large number of samples, plan on encapsulating 2-3-fold more cells than you wish to analyze, in order to have backups in case of unforeseen problems with library prep, and for use as technical replicates.

For example, if you feel that obtaining transcriptomes for 1,000 cells is sufficient, then you should collect 3,000 cells. If you are interested in 3,000 cells, collect 6,000 cells. If you want to look at 10,000 cells, consider using 2-4 biological samples of 5-10,000 cells each.

For cost reasons we would not advise collecting more than 20,000 to 30,000 cells per day, split over 1-8 samples. However, for initial experiments we advise testing 2-4 samples of collecting to 3,000 cells each. Sequencing results may reveal problems with sample preparation and it is therefore best not to use up too many reagents. Note that these numbers refer to the total number of cells to be barcoded. The actual number of cells required per sample is higher (see below).

Consider detailed plan for the inDrops experiment: even for small samples it is realistic to spend 20-30 minutes per sample. System prep (prior to your arrival) takes ~1½ hours. After the end of encapsulation, samples require an additional ~3h of processing time (for UV, RT, splitting, droplet breaking and freezing of emulsions).

Consider your sequencing budget: for deep sequencing, each 1,000-3,000 cells should be loaded on to a single lane and can cost $1500-$2100 to sequence. For some situations you may be satisfied with fewer reads and can then sequence up to 10-fold more cells/samples per lane. For initial experiments we strongly advise using just 1000-2000 cells per lane in order to gain good coverage before trying shallower coverage. The cost may limit the scale of your experiments.

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

While only 3-10k cells per sample might be encapsulated, we need to handle larger volumes of cells for input. inDrops can barcode 60-90% (typically 80%) of cells flowing into the inDrops device. It can process the entire volume of very rare samples, e.g. just a few thousand cells in total. But this takes more time and can lead to greater variability across the sample as cells start dying while on ice.

Please design your experiment to provide at least 25,000 cells per sample, even if you only want to barcode as little as 3,000 cells from this sample. This will reduce risk of losing the sample due to technical errors, and makes sample handling much simpler. The more cells you can provide the easier and faster your experiment will proceed.

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.

Try OptiPrep for maintaining single cell suspensions

Cells are diluted in roughly 15% OptiPrep solution before loading on InDrops. The exact percentage can change slightly with different cell types. OptiPrep is useful for removing dead cells from your sample prior to InDrops (see pages 5-6 on OptiPrep manual). However, if you had a high percentage of dead cells that are removed by OptiPrep you should check that the remaining viable cells are not contaminated by free-floating RNA released by the dead cells. See last section of this document for how to check for free-floating RNA.

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 100μL.

The minimum cell concentration we can work with is 150k/ml in 150ul, which is roughly 25,000 cells.

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. This raises the risk of cell death. While dead cells can be sorted based on their transcription profile in the final sequencing, this will waste reads, and more importantly dead cells might leak RNA leading to background noise for the whole experiment.

To test if cells are viable up until the moment of encapsulation, keep cells on ice for 1 hour after dissociation into the final buffer, then mix/resuspend the cells by pipetting. Inspect 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.

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

This test is optional but highly recommended for primary tissues.

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.