Samples preparation guidelines for 10X Genomics 3'GE scRNA-seq

This protocol for the classical 3'GE scRNA-seq serves as our main 10XG scRNA-seq guidelines. Additional short guidelines may be available for specific applications (5'GE VDJ, CellPlex, CRISPR screen, surface protein detection, targeted sequencing, scRNA-seq + scATAC-seq…).

Disclaimer

10XG scRNA-seq runs work in general well, but should still be considered at the edge of current technological capacities. There are indeed a small but significant percentage of wells/runs that fail for purely technical reasons. Of course, we don't charge for these unless we can spot a critical error from the user side (e.g. failure to follow cell prep guidelines/buffers), or if users gave us their green light to carry on despite poor quality cells/low viability. Importantly, if a putative failure would represent a major issue for your project (e.g. months-long mice generation to repeat), tell us in advance and we'll discuss the risks and means to minimize them. As a general rule, if you have extra cells, and if the experiment is critical, you can freeze them as a backup.

Scheduling the experiment

CAUTION: At least 3 weeks prior notice is needed for a regular scRNA-seq experiment, but more may be needed for more complex experiments (multiplexing…). In any case, do not start an experiment that requires samples collection on a specific week before having checked/scheduled with us.

Interactions with GECF on experiment day

IN SHORT: Bring your cells prepared and washed according to these guidelines, on ice, at 700-1200 cells/ul, in minimum 50ul, without EDTA. Bring medium for putative dilutions. Indicate number of targeted cells, and bsl2 level if relevant.

Targeted cell number

- Define the number of cells you want data for (="targeted cell number"), and indicate it to us at the latest when bringing cells. Recovery rate is uncertain and depends on physical characteristics of the cells, as well as on experiment-specific factors, such as too low/too high cell concentration, dead cells %, debris, etc. therefore targeted cells number is only indicative and number of actual recovered cells can differ significantly from it. If you absolutely want a given minimum number of cells, consider adding 20% safety margin to compensate for putative low capture efficacy.
- The rate of doublets increases with targeted cells number (see table below), therefore we recommend not targeting more than 5'000 cells unless absolutely necessary.

Nb of Recovered Cells	Multiplet Rate (%)
500	~0.4%
1 000	~0.8%
2 000	~1.6%
3 000	~2.3%
4 000	~3.1%
5 000	~3.9%

6 000	~4.6%
7 000	~5.4%
8 000	~6.1%
9 000	~6.9%
10 000	~7.6%

- Each additional cell comes with a sequencing cost, therefore calculate the number of cells needed thoughtfully. Once the cells have been processed, it is not possible to sequence only a fraction of them.
- The ideal number of targeted cells depends on the biological question. For comparing two populations, 1'000 cells may be enough. At the other extreme, for identifying new rare subpopulations of cells (<1%), 5'000 or more cells may be needed. If unsure, we recommend 4'000 cells as a good starting point.

Cells preparation

Cells must be submitted ready for subsequent steps in the procedure, according to the guidelines below and to the latest version of 10XG Cell Prep guide (document CG00053, https://support.10xgenomics.com/single-cell-gene-expression/sample-prep/doc/demonstrated-protocol-single-cell-protocols-cell-preparation-guide). This 10XG document explains how to wash and resuspend cells.

10XG have an online database for publications, searchable by organism and tissue, useful to find protocols for specific tissues: https://www.10xgenomics.com/resources/publications?query=&page=1

CAUTION: obtaining nicely dissociated and alive cells is likely the most important and often most tricky step of the whole 10XG experiment. Therefore, we strongly recommend that you test and optimize the dissociation procedure beforehand, including any putative step where the cells will have to wait on ice before being processed by us (waiting for other samples to be dissociated, trip towards GECF…).

Dissociation

- Perform at least one wash, as described in the 10XG cell prep guide. This wash can be performed with your culture medium. In case cell amount is too low, this can exceptionally be skipped (see cell prep guide).
- Cells should be brought either in PBS 0.04% BSA, or alternatively can be kept in their preferred medium (FCS is not a problem, as long as it was filtered to remove putative large particles). If using BSA, use a BSA stock of molecular biology grade or similar grade. 10XG recommend:
  - Millipore/Sigma, Bovine Serum Albumin In DPBS, #A1595
  - ThermoFisher Scientific, UltraPure Bovine Serum Albumin, #AM2616
- Place cells on ice once prepared.
- Cells should be well dissociated from each other, with minimal amounts of doublets. If relevant carefully gate on FACS to avoid doublets.
- Absolutely avoid aggregates or clumps as they may clog the capillaries and lead to run failure. To avoid these, cells can be passed through a cell strainer. This is usually not necessary if cells are FACS-sorted. Avoid doing that if it comes with a risk of skewing your data. Some examples:
  - Flowmi pipette cell strainer of 40um or 70um (we have both at GECF).
  - Miltenyi Biotec 30 um PreSeparation Filter, cat#130041407….

- Avoid cell debris as much as possible, as they are encapsulated along with cells and impact data negatively.
- If used, trypsin should be inactivated after use (serum, BSA…).
- EDTA is not OK here as it will impede reverse transcription. If you use EDTA to detach cells, quench it and do an additional wash. If really needed, limit it to 0.5mM, but still without guarantee of success. EGTA is better as it mostly chelates Ca++ rather than Mg++.
- Notes
  - For dissociation of difficult tissues, refer to publications from the relevant field, contact 10XG tech support, look at users-developed protocols (https://community.10xgenomics.com/t5/Customer-Developed-Protocols/ct-p/customer-protocols) and/or refer to 10XG webpage containing useful links: https://kb.10xgenomics.com/hc/en-us/articles/218169563-How-do-I-dissociate-my-tissue-of-interest , such as the Worthington Tissue Dissociation Guide. Using a GentleMACS instrument may help, as well as accutase, collagenase, pronase or even better psychrophilic proteases which work at 4° (Potter lab, PMID: 28851704).
  - Some people recommend DNase 1 treatment. For instance: https://www.stemcell.com/how-to-reduce-cell-clumping-in-single-cell-suspensions-with-dnase.html . Cells/tube walls must be especially well washed if used.
  - As a general rule, remember that expression profiles can change rapidly if dissociation is lengthy, in particular if not performed at cold temperatures. If you fear the dissociation procedure may alter the cell state, you can collect the dissociated cells suspension, extract RNA and ask us to perform a bulk RNA-seq on these in parallel to the undissociated tissue of origin.
  - When obtaining clean single cells suspension is not possible, isolated nuclei can be run instead of cells. 10XG have several protocols on their website. The CryoPrep system from Covaris can also be used to isolate nuclei from difficult to dissociate tissues.
  - Some tissues are specially RNases-rich, in particular pancreas, spleen and lungs (https://kb.10xgenomics.com/hc/en-us/articles/4415486278669-Can-I-use-RNase-rich-tissue-samples-for-single-cell-gene-expression-or-Multiome-assays-). With such tissues, in particular many cells are dying and release their content, it is worth adding 0.2-1U/ul of RNase inhibitor in the final resuspending buffer/medium. 10XG recommend Sigma-Aldrich PN-3335399001. CAUTION: some inhibitors impact negatively performances:

Other RNase inhibitors tested and shown to negatively impact data quality:

- SUPERaseIn RNase Inhibitor
- Ribonucleoside Vanadyl Complex

It is strongly recommended to use the RNase Inhibitor part number listed in the Multiome ATAC + Gene Expression nuclei isolation demonstrated protocols (Sigma Protector RNase inhibitor, PN-3335399001).

Ambion™ RNase Inhibitor (AM2684 Thermo Fisher Scientific), RNaseOUT™ Recombinant Ribonuclease Inhibitor (10777019 Thermo Fisher Scientific) and, RiboLock RNase Inhibitor (Thermo Fisher, PN-EO0382) may be used as an alternative when Sigma Protector RNase inhibitor is not available. Note: there was limited testing of these inhibitors for Multiome ATAC + Gene Expression, so use with caution.

Cell number and concentration

- Cells concentration should be 700-1’200 cells /ul (incl dead cells). Concentrations outside this range makes recovered cell number even less predictable than it is intrinsically. FACS often overestimate cells concentration, therefore take some safety margin and ask the sorting facility to give you a concentration a bit higher than that.
- We need > 50ul to perform the QC and the run itself in good conditions for any number of targeted cells. If no more than 5’000 cells are targeted, 25ul could still be manageable if you cannot get more. If these values cannot be reached, contact us in advance.
- For concentrating or washing cells, 10XG recommend spinning at RT° at 300xg for 5min for small cells (5-10um), 250xg for 4min for medium cells (10-17um), and 150xg for 3 min for larger cells (17-25um). This insures efficient pelleting while minimizing carryover of debris or aggregation of cells. When doing it the first time, keep and check also supernatant for absence of cells. If you have or suspect to have a sub-population composed of small cells, prefer small cells settings to avoid losing it.
- Before loading cells in the instrument, we'll double-check the cell concentration.
- Bring some extra medium to perform cells dilutions if needed.
- When cells tend to aggregate, cell concentration should be kept on the lower side of the range (aim for 700 cells/ul rather than 1200 cells/ul).
- Automated counter such as the Countess are generally not accurate for very small cells.

Viability

- The key word for the 10XG Chromium is cell viability. It is very important to get good results downstream.
- Dead cells can generally be excluded bioinformatically as they contain a high percentage of mitochondrial RNAs, but it may not be done easily if cells are in the process of dying. The percentage of dead cells that is considered acceptable depends on your experiment. If your cells come from a healthy suspension cell line, anything more than 10% dead cells is probably a bad sign, while if working from primary cells that underwent hours of dissection and sorting, 20% may be considered acceptable. We will move forward with any sample containing less than 20% dead cells, unless the user explicitly decides otherwise. When samples contain more dead cells, it is the user/PI decision to either move forward anyway, or perform a dead cells removal procedure (see below), or cancel the experiment.
- MACS-sorting in general leads to higher viability than FACS-sorting, so prefer the former if possible. If FACSing, prefer larger nozzle and lower pressure (discuss ahead with flow facility team)
- If you sort the cells by FACS before loading, include a viability stain to get rid of dead cells if possible.
- Miltenyi offer a kit to remove dead cells if needed (“Dead Cell Removal kit”, 130-090-101), which works at least on mammalian cells (probably also on insect cells but to be confirmed).
- We'll check viability of the cells before loading them in the instrument.

Diverse important notes

Number of reads/cell

The choice of number of reads/cell impacts costs and therefore can influence how many cells you target, it is thus

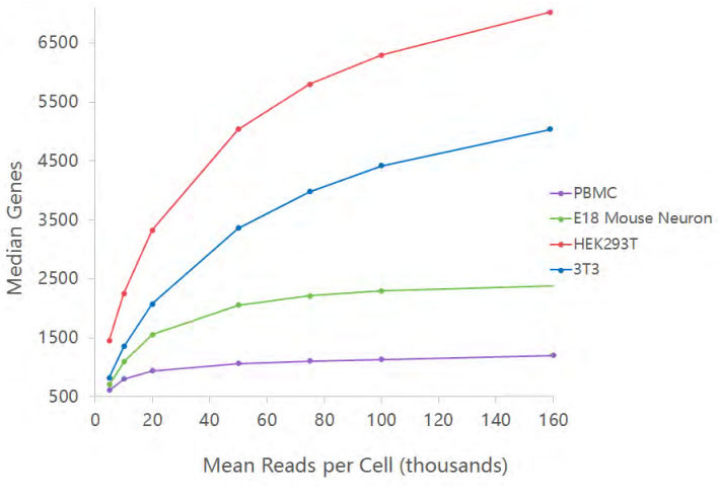
good to have a rough idea already before processing the cells. There are 3 factors that can influence this choice:

- the size of the cells. For very small cells (5-10um), mRNA levels are low and sequencing saturates fast, thus 50-100k reads/cell capture most of the available information. In contrast big cells (>25um) contain a lot of mRNA can be sequenced up to 200k reads/cell and above and still capture new information.
- the biological question: for clustering cells in groups of known cell types, and/or to delineate their broad transcriptional profile, 50k reads/cells is enough (even for big cells). If you want to zoom on specific genes, then the more reads the better (up to complete sequencing saturation, see above). If you also want to delineate new cell type with subtle differences, having more reads is also better.
- the amount of money you are willing to spend, which also depends on the number of samples and cells.

If you're not sure, 10XG recommend 50k reads/cells as a starting point. We rather go for 75k as a starting point.

Cell size

- Max cell diameter supported by 10XG is 30um, but the max theoretical limit is 65um (=size of the microcapillaries). These diameters are calculated for cells in suspension, not flattened on a petri dish.
- In case your cells are too big, a special protocol exists for nuclei instead of cells (see above).
- Number of genes detected directly depends on the cell size (=amount of mRNA molecules per cell). Sequencing depth chosen may thus be modulated according to cell size. See plot from 10XG (for v2 reagents):



Random notes

- A general overview of 10XG experimental design: https://pages.10xgenomics.com/3p-getting-started-guide-single-cell-gene-expression.html
- Special protocols allow to measure cell surface protein levels or monitor CRISPR perturbation screens along to the usual scRNA-seq. More information here: https://support.10xgenomics.com/single-cell-gene-expression/overview/doc/getting-started-single-cell-gene-expression-with-feature-barcoding-technology
- The standard protocol is intended for fresh unfixed/unfrozen cells, but a special protocol exists for methanol-fixed cells. Inquire if interested.
- If an mRNA from an ectopically-expressed transgene is highly overexpressed (GFP…) it may take a significant fraction of the reads, therefore take that into account when designing your experiment.

- Samples must not contain toxic/carcinogenic chemicals. Contact us in advance if it is not possible.
- If TCR/BCR VDJ-seq is needed, tell us in advance as the whole method is different. See separated guidelines.
- If you have to prepare cells in different batches, make sure you avoid batch effects. Typically, avoid processing all controls in a batch and "treated" samples in a distinct batch.
- We can split the processing of cells in the instrument in several batches if this avoids some samples waiting on ice for very long times, and if this does not introduce batch effects.
- If you plan a big experiment, it may be worth first running just a single sample (pilot experiment) to assess quality of data and cell recovery rate.
- 10XG regularly update their reagents and workflows/pipelines. If you absolutely want us to use a specific version for comparing with a previously generated dataset, tell us ahead and we'll discuss what can be done. Yet we recommend to always use the latest version as improvements are often significant.
- When processing PBMC/blood cells, absence of left-over/contaminating erythrocytes is important to avoid losing reads to their ultra-abundant globin mRNA. In the same manner, if the isolation protocol includes an erythrocytes lysis step, it is critical to perform very careful washes afterwards to remove their floating mRNAs.
- Biosafety:
  - We have 2 instruments, one on a regular bench and one under a bsl2 hood. We can therefore process bsl2-level samples, but please warn us before the experiment day. Primary human cells must have been screened for absence of HIV, HBV and HCV infection (ideally also hCMV), or they will be considered bsl2 material. More generally, cells must not be infected with an infectious agent.
  - To determine the biosafety level of cell lines, this German website is used as a reference by the Swiss Biotechnology Office (the website is from the German government actually): https://zag.bvl.bund.de/zelllinien/index.jsf?dswid=7026&dsrid=373 . A few cell lines are also listed here: https://www.bafu.admin.ch/dam/bafu/en/dokumente/biotechnologie/fachinfo-daten/einstufung_von_organismenzelllinien.pdf.download.pdf/einstufung_von_organismenzelllinien.pdf
  - When nuclei are used, the isolation procedure may be sufficient to declassify your cells from bsl2 to bsl1, please investigate with us if relevant.
- Neutrophils can be difficult to detect with 10XG method, see here for details (https://kb.10xgenomics.com/hc/en-us/articles/360004024032-Can-I-process-neutrophils-using-10x-Single-Cell-applications) and warn us in advance if this cell type is important for you.
- 10XG released a samples multiplexing system called CellPlex. See our separate guidelines for details.
- If the bioinformatics analysis is to be performed at GECF, tell us in advance in case an ectopically expressed gene must be added to the reference genome. In particular, tags/markers that are inserted in 3'/C-ter of endogenous ORFs will require special thoughts, as the reads will likely lie within these added parts rather than the endogenous gene, and mapping on the endogenous gene will give no hit. Also, if the transgene is expressed from an integrated lentivector, the 3'UTR extends until the 3'LTR and the reads will be there.
- Droplets containing multiple beads occur at a mean frequency of ca 4%, and mRNAs from cells captured in these droplets are split into these multiple beads, hence will be detected as multiple cells, each with low UMIs. It is very likely that such "ghost cells" are filtered out during data processing, but to be on the safe side, new rare cell population composed of cells with low UMI counts should be validated by a different method.
- A Low Throughput version of the reagents is available for pilot studies (max 1'000 cells, degraded specs).
- This is a polyA-based method therefore won't catch non-polyA RNAs.

Versions log

- vA.01-vA.11: earlier releases.
- vA.12: Clarified that 10XG regularly update their reagents/pipelines, and that if you absolutely want us to use a specific version to be able to directly compare with one of your previously generated dataset, tell us ahead. Clarified that, ectopic tags/markers that are inserted in 3'/C-ter of endogenous ORFs require special thoughts, as the reads will likely lie within these added parts rather than the endogenous gene. Clarified how to determine ideal number of targeted cells, and ideal number of reads/cells.
- vA.13: Indicated that neutrophils are difficult to detect. Mentioned the ghost cells issue, and ways to take that into account. Indicated to pay special attention to erythrocytes when preparing PBMCs/blood cells. Commented on minimum number of cells to bring. Clarified that when the transgene is expressed from a lentivector, the reads will be in the 3'LTR. Described specific procedures in case of bsl2 operations. Suggested to do bulk RNA-seq at the end of the dissociation procedure in case the procedure is harsh and may impact on cell states. Introduced CellPlex. Commented on negative impact of cell debris and clumps.
- vA.14: Mentioned new low throughput reagents. Further considerations regarding biosafety. Clarified EDTA absence. Mentioned to use low pressure/large nozzle for FACS. Mentioned DNase 1 treatment for cells dissociation. Mentioned that only polyA RNAs are sequenced. Mentioned searchable publications database. Mentioned which BSA can be used to prepare the PBS 0.04% BSA. Added an important disclaimer section.
- vA.15: Clarified not to schedule an experiment without our prior agreement regarding a timeframe. Suggested the use of RNase inhibitors in some cases.