10x Genomics Multiome (scRNA-seq/scATAC-seq) Guidelines

Workflow of interactions with GECF

- **IN SHORT:** At least 3 weeks in advance, schedule the experiment with us and read these guidelines. Few days in advance, send us the submission form (to be found on our website). On experiment day, bring your nuclei, on ice, prepared and washed according to the specific relevant 10XG protocol and using your previously-optimized lysis conditions/duration, at the concentration relevant for your targeted nuclei number (see below), in ideally minimum 25ul, resuspended in the Diluted Nuclei Buffer that we will give you. Tell us number of targeted nuclei, and bsl2 level if relevant. If possible, also bring an aliquot of unlysed cells so we can check their viability.

Targeted nuclei number

- Define the number of nuclei you want data for (=“targeted nuclei number”) and indicate it in the submission form. Recovery rate is uncertain and depends on characteristics of the nuclei, as well as on experiment-specific factors, such as debris, etc. therefore targeted nuclei number is only indicative and number of actually recovered nuclei can differ significantly. If you absolutely want a given minimum number of nuclei, indicate it in the submission form and we will add a safety margin. In addition, since a cell can be successfully called only if both scRNA-seq and scATAC-seq were successful for that cell, the number of called cells is lower than in regular 10XG scRNA-seq, and you may want to apply an extra safety margin to the targeted cells number.
- The rate of doublets increases with targeted nuclei number (see table below), therefore we recommend not targeting more than 5’000 nuclei unless really necessary.

<table>
<thead>
<tr>
<th>Targeted Nuclei</th>
<th>Multiplet Rate (%)</th>
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<tbody>
<tr>
<td>500</td>
<td>~0.4%</td>
</tr>
<tr>
<td>1 000</td>
<td>~0.8%</td>
</tr>
<tr>
<td>2 000</td>
<td>~1.6%</td>
</tr>
<tr>
<td>3 000</td>
<td>~2.3%</td>
</tr>
<tr>
<td>4 000</td>
<td>~3.1%</td>
</tr>
<tr>
<td>5 000</td>
<td>~3.9%</td>
</tr>
<tr>
<td>6 000</td>
<td>~4.6%</td>
</tr>
<tr>
<td>7 000</td>
<td>~5.4%</td>
</tr>
<tr>
<td>8 000</td>
<td>~6.2%</td>
</tr>
<tr>
<td>9 000</td>
<td>~6.9%</td>
</tr>
<tr>
<td>10 000</td>
<td>~7.7%</td>
</tr>
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</table>

- Each additional nucleus comes with a sequencing cost, therefore calculate the number of nuclei needed thoughtfully. Once the nuclei have processed, it is not possible to sequence only a fraction of them.
- The ideal number of targeted nuclei depends on the biological question. For comparing two populations, 1’000 nuclei may be enough. At the other extreme, for identifying new rare subpopulations (<1%), 5’000 or more nuclei may be needed. If unsure, we recommend 3’000-4’000 nuclei as a good starting point.
Nuclei preparation

Nuclei must be submitted ready for subsequent steps in the procedure, according to the guidelines below.

Nuclei isolation

− CAUTION: obtaining nicely dissociated, healthy nuclei with a minimum number of unlysed cells is likely the most important and often most tricky step of the experiment. Therefore, we strongly recommend that you test and optimize the nuclei isolation procedure beforehand, including any putative step where the nuclei will have to wait on ice before being processed by us (waiting for other samples, journey towards GECF...).
− There is no universal nuclei isolation protocol, therefore, 10XG recommend you choose among these options depending on your cell type:
  • Look in the literature for publications performing 10XG multiome analysis on exactly your cell type and test this protocol. If your protocol of choice has not been specifically validated for 10XG multiome, submit it to us and/or 10XG tech support for review, and test it in advance.
  • 10XG released in 2022 a nuclei isolation kit (https://www.10xgenomics.com/support/single-cell-atac/documentation/steps/sample-prep/chromium-nuclei-isolation-kit-sample-prep-user-guide), we have it in house and can give you aliquots for you to try.
  • 10XG released protocols for some specific cell types or tissues, which you can check out or base yourself on: Google for the latest version of 10XG documents # CG000365, CG000366, or CG000375.
− If your protocol has not been validated exactly for your cell type, it can be required to perform an optimization of both duration of lysis (e.g. 2/4/6/8), and concentration of lysis buffer (e.g. 0.3x, 0.6x, 1x): https://kb.10xgenomics.com/hc/en-us/articles/360053165711-How-do-I-perform-a-lysis-timeline-to-optimize-my-nuclei-isolation-for-Single-Cell-Multiome-ATAC-Gene-Expression-. Make sure to dilute the lysis buffer in the specific buffer mentioned, not in water. Finally perform the optimization with nuclei concentration that you will use for your experiment, as this can impact on aggregation behavior. If you are using the protocol for Nuclei Isolation from Complex Tissues (document CG000375), please note that lysis time course and buffer strength optimization are only referring to nuclei isolation step in NP40 lysis buffer (10XG do not recommend altering the 0.1x Lysis Buffer used for nuclei permeabilization).
− Isolated nuclei should be intact/healthy, with clear edges and no significant blebbing:

− Many isolation protocols require a high amount of RNase inhibitor. If you have many samples, the RNase inhibitor recommended by 10XG (“Protector”) may become prohibitively expensive. In that case check the alternatives mentioned at the bottom of that page: https://kb.10xgenomics.com/hc/en-us/articles/360049543672-Can-I-use-an-alternative-RNase-inhibitor-part-number-
− Consider that the nuclei isolation procedure will likely lead to ca 50% nuclei compared to starting cell number.
− In case the starting number of cells is low, the nuclei isolation protocols of 10XG have in general specific guidelines, such as less washes. Follow them if relevant.
− The nuclei prep should not contain more than 5% alive cells. These will indeed cluster separately in the scRNA-
Avoid cell debris as much as possible, as they are encapsulated along with nuclei and impact data negatively.

Nuclei must be brought in the Diluted Nuclei Buffer that we will give you on the day of the experiment. We’ll arrange for that beforehand. CAUTION: to save on RNase inhibitor costs, we’ll give you enough to resuspend the nuclei in 200ul buffer final for each sample, with some significant extra safety margin. If this volume is not sufficient for the amount of nuclei you have, you’ll have 2 solutions: 1) before the final centrifugation, split your nuclei and spin only a fraction consistent with the available 200ul of buffer; 2) spin and resuspend everything in the dedicated 200ul then dilute a small aliquot of that into 30ul final to reach the desired concentration (you will have enough volume for that).

Absolutely avoid aggregates or clumps as they may clog the capillaries and lead to run failure. To avoid these, nuclei can be passed through a cell strainer. Some examples:
- Flowmi pipette cell strainer of 40um or 70um (we have both at GECF).
- Miltenyi Biotec 30 um PreSeparation Filter, cat#130041407….

Nuclei should be well dissociated from each other, with minimal amounts of doublets. If performing FACS, carefully gate on FACS to avoid doublets.

If used, trypsin should be inactivated after use (serum, BSA…).

If you use custom resuspension buffer (not recommended), it must be devoid of EDTA.

Place nuclei on ice once prepared.

Notes
- The CryoPrep system from Covaris can also be used to isolate nuclei from difficult to dissociate tissues.
- Unless specifically agreed by 10XG/us, do not stain the nuclei with a DNA intercalating dye, as it is likely to impact the ATAC procedure. The 7AAD dye is the only one that has been approved for now by 10XG.
- For concentrating/washing cells before nuclei isolation, 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.
- Frozen cells can be used as a starting material for nuclei isolation, leading to a modest loss of data quality (10XG FAQ section of website). Nuclei cannot be frozen.
- FACS may be needed/useful to remove debris after nuclei isolation.
- If assessing nuclei quality is tricky, a fluorescent stain such as DAPI can be used to differentiate them from debris (on an aliquot of nuclei).
- If it is suspected that the cells suspension contains a lot of ambient DNA, it is possible to treat cells with DNase (at the cells step only, before the nuclei isolation procedure/washes).
Summary slide from 10XG regarding their isolation recommendations:

- **Nuclei number and concentration**
  - The concentration of the nuclei you bring must be in the following ranges, which depend on the number of nuclei you ultimately want data for (“targeted nuclei recovery”). On the right are recommendations for concentration to aim for, balancing the need for a concentration suitable for accurate counting, a safety margin to avoid having to spin again, while avoiding too high concentrations which favor clumps/doublets:

<table>
<thead>
<tr>
<th>Targeted Nuclei Recovery</th>
<th>Nuclei Stock Concentration (nuclei/μl)</th>
<th>Aim for (nuclei/μl):</th>
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<tbody>
<tr>
<td>500</td>
<td>160-400</td>
<td>400</td>
</tr>
<tr>
<td>1,000</td>
<td>320-810</td>
<td>800</td>
</tr>
<tr>
<td>2,000</td>
<td>650-1,610</td>
<td>1,000</td>
</tr>
<tr>
<td>3,000</td>
<td>970-2,420</td>
<td>1,400</td>
</tr>
<tr>
<td>4,000</td>
<td>1,290-3,230</td>
<td>1,900</td>
</tr>
<tr>
<td>5,000</td>
<td>1,610-4,030</td>
<td>2,400</td>
</tr>
<tr>
<td>6,000</td>
<td>1,940-4,840</td>
<td>2,700</td>
</tr>
<tr>
<td>7,000</td>
<td>2,260-5,650</td>
<td>2,900</td>
</tr>
<tr>
<td>8,000</td>
<td>2,580-6,450</td>
<td>3,100</td>
</tr>
<tr>
<td>9,000</td>
<td>2,900-7,260</td>
<td>3,300</td>
</tr>
<tr>
<td>10,000</td>
<td>3,230-8,060</td>
<td>3,500</td>
</tr>
</tbody>
</table>

- Ideally we need >25μl of nuclei at the right concentration to perform the QC and the run itself in good conditions. If you suspect these values cannot be reached, contact us in advance. If this is not possible to reach, less is also possible, down to ca 7-8μl but rendering the nuclei QC process much trickier.

- The loading of the nuclei and calculations are very different for multiome than for other 10XG methods, therefore do not apply here guidelines that were given to you in other contexts.

- If FACS is used, consider that FACS often overestimate cells/nuclei concentration, therefore take some safety margin and ask the sorting facility to give you a concentration a bit higher than aimed at.
− Make sure your counting device works with small nuclei. If unsure, Neubauer chambers are recommended.

Viability
− For a successful 10XG Multiome experiment, cell viability must be high before starting the nuclei isolation. Indeed, with nuclei, it is difficult to exclude dying/dead cells bioinformatically as this relies on mitochondrial RNAs, which are absent from nuclei. 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 with primary cells that underwent hours of dissection and sorting, 20% may be considered acceptable. 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.

− If possible, bring an aliquot of unlysed cells so we can check their viability.

− 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 nuclei isolation, 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).

Diverse notes
− The CellPlex samples multiplexing solution is not compatible with multiome. An alternative solution for multiplexing is to use BioLegend TotalSeqA barcoded antibodies against nuclear surface proteins. This is not supported officially though, and demultiplexing will have to be done by a bioinformatician on your side.

− If you have to prepare nuclei 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 nuclei 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 is strongly recommended first running just a single sample (pilot experiment) to assess quality of data and nuclei 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.

− Biosafety:
  • We will process bsl2-level samples under a bsl2 hood. Please warn us before the experiment day.
  • Primary human cells that have not been screened for absence of HIV, HBV and HCV infection (ideally also hCMV), are considered bsl2 material.
  • To determine the biosafety level of cell lines, this German website is used as a reference by the Biotechnology office of the Confederation: 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/
  • Since 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.

- If many granulocytes are present, it is recommended to sort them out as they may perform netosis which affects ATAC-seq data quality.

- For the bioinformatics analysis, tell your bioinformatician 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 UMI.s. 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.

- The number of reads/nuclei you want depends on the biological question: for clustering cells in groups of known cell types, and/or to delineate their broad transcriptional/chromatin profile or activated pathways, 25k reads/nuclei is enough. If you want to zoom and ask whether specific genes are expressed, then the more reads the better (up to complete sequencing saturation, which is likely between 50k and 100k for nuclei).

### Versions log

- vA.01-vA.04: Clarified the volumes of nuclei resuspension that will be given to users. Commented on the higher safety margin needed for multiome for targeted cell numbers as compared to isolated scRNA-seq and scATAC-seq applications. Indicated to bring unlysed cells to assess viability.

- vA.05: Clarified that calculations/loading for multiome are very different than for 3’/5’GE and therefore guidelines from these applications cannot be transposed without our approval. Clarified that for nuclei isolation from complex samples, the optimization is only relative to NP40 lysis buffer and not to the nuclei permeabilization. Clarified that we load at max 5 ul of nuclei. Clarified the nuclei number and concentration.