



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

Interactions with GECF on experiment day

- **IN SHORT:** 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 to us at the latest when bringing nuclei. Recovery rate is uncertain and depends on physical 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 actual recovered nuclei can differ significantly from it. If you absolutely want a minimum given number of nuclei, consider adding 20% safety margin to compensate for putative low capture efficacy. *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 often lower than in regular 10XG scRNA-seq. Therefore, you may want to apply a bigger safety margin to the number of targeted cells.*
- The rate of doublets increases with targeted nuclei number (see table below), therefore we recommend not targeting more than 5'000 nuclei unless absolutely necessary.

| Targeted Nuclei | 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 | ~6.2% |
| 9 000 | ~6.9% |
| 10 000 | ~7.7% |

- 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 nuclei as a good starting point.

Nuclei preparation

Nuclei must be submitted ready for subsequent steps in the procedure, according to the guidelines below and to the latest version of the relevant 10XG Nuclei isolation protocol for Multiome (document [CG000365](#), [CG000366](#), or [CG000375](#), or similar protocols validated for 10XG multiome). These protocols address isolation from both cell lines and tissues. If your protocol of choice has not been specifically validated for 10XG multiome, we strongly recommend that you submit it to us and/or 10XG tech support in advance for review.

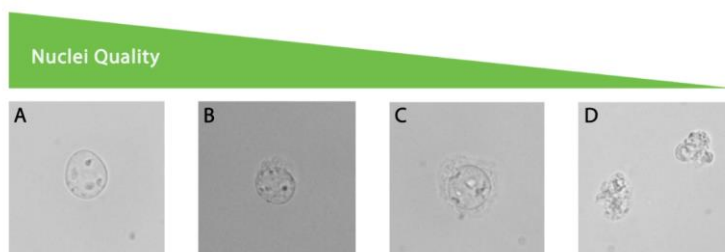
CAUTION: these protocols require a high amount of RNase inhibitor, among other reagents, make sure you have these. If you have many samples, the recommended Protector RNase inhibitor may become prohibitively expensive. In that case check the alternatives mentioned at the bottom of that page:

<https://kb.10xgenomics.com/hc/en-us/articles/360049543672-Can-I-use-an-alternative-RNase-inhibitor-part-number->

CAUTION: obtaining nicely dissociated, healthy nuclei with a minimum number of intact 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 to be dissociated, trip towards GECF...).

Nuclei isolation

- 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.
- It is crucial to perform an optimization of both duration of lysis (e.g. 2/4/6/8, or 2/3/4/5 minutes depending on fragility of the cells/nuclei), 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.*
- The nuclei prep should not contain more than 5% alive cells. These will indeed cluster separately in the scRNA-seq and complicate the analysis.
- Isolated nuclei should be intact/healthy, with clear edges and no significant blebbing:



A & B: OK to proceed. C: “at your own risk”. D: Do not proceed.

- 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 pellet only a fraction consistent with the available 200ul volume of buffer; 2) pellet and resuspend everything in the dedicated 200ul and 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....
- Avoid cell debris as much as possible, as they are encapsulated along with nuclei and impact data negatively.
- 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 as it will impede downstream reactions.
- Place nuclei on ice once prepared.

- Notes
 - For dissociation of difficult tissues, refer to publications from the relevant field, contact 10XG tech support, and/or refer to 10XG webpage containing useful links: <https://kb.10xgenomics.com/hc/en-us/categories/360004142131-Single-Cell-Multiome-ATAC-Gene-Expression>.
 - 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.
 - Useful further tips from 10XG: <https://kb.10xgenomics.com/hc/en-us/articles/360050490472-What-are-the-best-practices-for-nuclei-isolation-for-Single-Cell-Multiome-ATAC-and-Gene-Expression->
 - 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 (not nuclei) can be used as a starting material, leading to a modest loss of data quality (10XG FAQ section of website).
 - 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).
 - FAQs are available here: <https://kb.10xgenomics.com/hc/en-us/categories/360004142131-Single-Cell-Multiome-ATAC-Gene-Expression>
- Summary slide from 10XG regarding their isolation recommendations:

Recommendations for optimizing nuclei isolation

| Lysis Buffer | Lysis Time | Wash & Spin | Debris Removal | Count and QC |
|---|---|--|--|--|
| Adjust concentration | Perform lysis timeline | Adjust spin conditions | Clean up suspension | Always assess final suspension |
| <ul style="list-style-type: none"> 1X Lysis Buffer for cells in suspension 0.1X Lysis Buffer for fragile tissues and tissue-derived cells | <ul style="list-style-type: none"> Broad timeline for tough cell types Refined timeline for fragile cells Aim for <5% viability | <ul style="list-style-type: none"> Increase spin time Use a swing-bucket rotor | <ul style="list-style-type: none"> Filter using Flowmi Use less stringent lysis buffer | <ul style="list-style-type: none"> Use Countess or hemocytometer with viability dye Use a fluorescent dye for increased accuracy Assess nuclear membrane under microscope without dye |

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:

| Targeted Nuclei Recovery | Nuclei Stock Concentration (nuclei/μl) | Aim for (nuclei/ul): |
|--------------------------|--|----------------------|
| 500 | 160-400 | 400 |
| 1,000 | 320-810 | 800 |
| 2,000 | 650-1,610 | 1000 |
| 3,000 | 970-2,420 | 1400 |
| 4,000 | 1,290-3,230 | 1900 |
| 5,000 | 1,610-4,030 | 2400 |
| 6,000 | 1,940-4,840 | 2700 |
| 7,000 | 2,260-5,650 | 2900 |
| 8,000 | 2,580-6,450 | 3100 |
| 9,000 | 2,900-7,260 | 3300 |
| 10,000 | 3,230-8,060 | 3500 |

- We ultimately can load max 5ul of cells in the instrument, but ideally we need >25ul 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 7-8ul but rendering the nuclei QC process much trickier.
- IMPORTANT: the loading of the nuclei and calculations are very different for multiome than for classical 3’GE/5’GE, therefore do not apply to multiome guidelines that were given to you in another context.

- Consider that the nuclei isolation procedure will likely lead to ca 50% nuclei as compared to starting cell number.
- 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

- Even though, nuclei loaded for multiome analysis are technically “dead”, the key word for a successful 10XG Chromium is good cell viability before starting the nuclei isolation.
- With nuclei, it is difficult to exclude dying/dead cells bioinformatically as this usually 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 from 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, please 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 important notes

Number of reads/nuclei

The choice of number of reads/nuclei 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. These are the factors to consider:

- the biological question: for clustering cells in groups of known cell types, and/or to delineate their broad transcriptional 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 no much more than 50k for nuclei). If you also want to delineate new cell type with subtle differences, more reads is also better.
- the amount of money you are willing to spend, which also depends on the number of samples and nuclei.

Random notes

- 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.
- The CellPlex samples multiplexing solution is not compatible with multiome.
- 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 may be worth 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. Yet we recommend to always use the latest version as improvements are in general significant.
- 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 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 (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/
 - 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.
- If the bioinformatics analysis is to be performed at the 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.

Versions log

- vA.01-vA.03: Clarified the volumes of nuclei resuspension that will be given to users.
- vA.04: 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.