protocol vA.12

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

Workflow of interactions with GECF

- **Schedule the experiment** with us at least **3 weeks in advance** (tell us already nb of samples and bsl2 level). Envision doing a 1-sample pilot before large experiments.

- **Optimize nuclei isolation procedure** (lysis conditions and time). We can check the nuclei.

- Few days in advance, send us the **submission form** (to be found on our website).

- **On experiment day**

  - bring your **nuclei on ice**, without DNA stain (7AAD tolerated)
  - prepared and washed according to the relevant 10XG protocol
  - at the **concentration** relevant for your targeted nuclei number,
  - in ideally **minimum 25ul**, resuspended in the **Diluted Nuclei Buffer** that we will prepare and give to you on the morning of the experiment.
  - If possible, also bring an aliquot of unlysed cells so we can check their viability, otherwise calculate cell viability yourself and let us know.

This method can give great results but it is very **difficult to evaluate nuclear integrity** just by looking at nuclei at the microscope. If small holes are present on the nuclear membrane, they will not be visible, but the RNA will leak out. Therefore before running a real experiment with multiple samples, we advise to do either

- a cheaper **pilot experiment** with only one sample
- **test RNA quality/quantity** by preparing bulk cDNA (cheaper option than a pilot). You can prepare nuclei as for the multiome, hand them to us and we will prepare cDNA and run Qubit and TapeStation on it.

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Targeted nuclei number

- Define the number of nuclei you want data for ("**targeted nuclei number**"). 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**.
- If you absolutely want a given minimum number of nuclei, indicate it in the submission form and we will add a safety margin in our calculations.
- A **cell/nucleus can be successfully called only if both scRNA-seq and scATAC-seq are good for that nucleus**, the number of called nuclei is therefore often lower than in regular 10XG scRNA-seq, and you may want to apply an extra safety margin to the targeted nuclei number.
- Given the tendency of nuclei to aggregate, we encourage not to push the method too close to its upper limit of 10k nuclei/sample unless really necessary.
- The **rate of doublets** increases with targeted nuclei number (see table below), therefore we recommend against targeting more than 5'000 nuclei unless really 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%

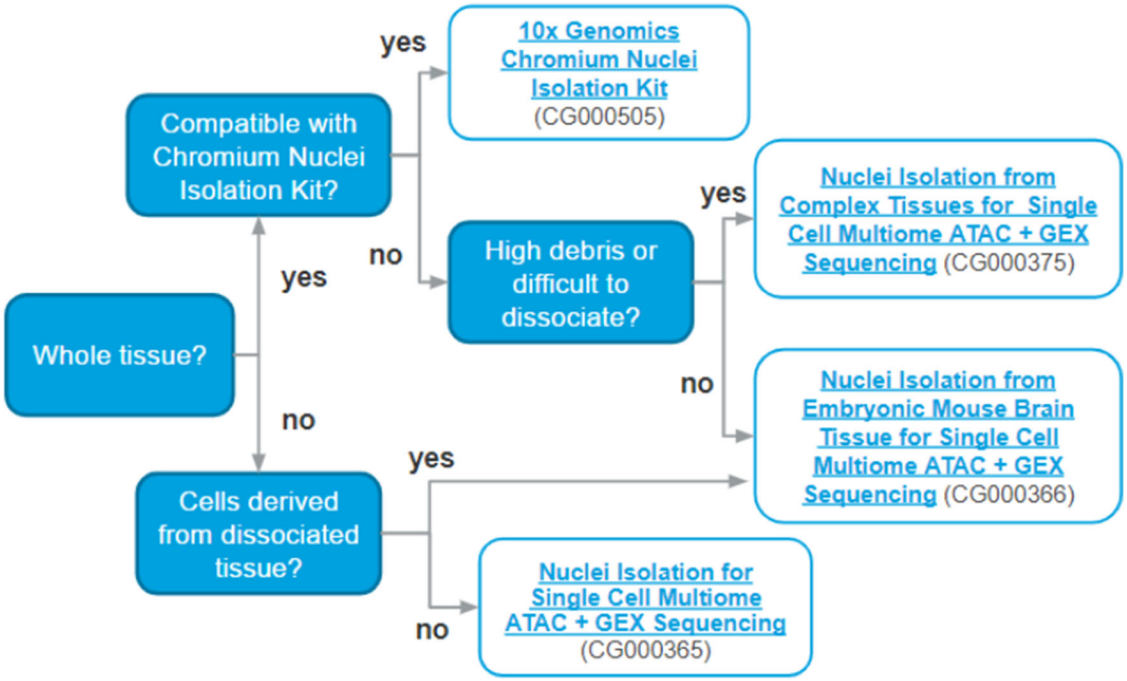
- Each additional nucleus comes with a **sequencing cost**, therefore calculate the number of nuclei needed thoughtfully. **Once the nuclei have been processed, it is <u>not</u> possible to sequence only a fraction of them**.
- The **ideal number of targeted nuclei** depends on the biological question. 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

Obtaining nicely dissociated, healthy nuclei with a minimum number of unlysed cells is the most important and often trickier step of the experiment. Therefore, we strongly recommend that you **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, trip towards GECF…).

- There is no universal **nuclei isolation protocol**, therefore, 10XG recommend you choose among these options

depending on your cell type:



- We have in house the **10XG Nuclei isolation kit** (**CG000505**) and we can sell aliquots.
- 10XG recommends **specific nuclei isolation protocols** for the different applications (scRNA, scATAC or scMultiome). For optimal assay performance, nuclei isolation should be performed using the protocol suggested for the specific application.
- If none of the above protocols are satisfying, look in the literature for publications performing 10XG multiome analysis on your exact cell type. If the protocol has not been specifically validated for 10XG multiome, submit it to us and/or 10XG tech support for review, and test it in advance.
- **Test in advance the chosen nuclei isolation protocol**. It may require an **optimization of both duration of lysis and concentration of lysis buffer**: 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. Choose the lowest strength/time that works well, because **overlysed nuclei leak transcripts**, leading to high ambient RNA and low GEX data complexity.
- **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), note that lysis time and buffer strength optimization only refer to nuclei isolation step in NP40 lysis buffer (10XG do not recommend altering the 0.1x Lysis Buffer used for nuclei permeabilization).*
- Regarding **RNase inhibitor:**
  - If using the Nuclei isolation kit, use the included RNase inhibitor.
  - For other protocols, the inhibitor recommended by 10XG ("Protector") may become prohibitively expensive if you have many samples. 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- . Use it at 1U/ul unless specified otherwise in your protocol.

- Number of **starting cells**:
  - 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 fewer washes. Follow them if relevant.
- **Don't over-spin nuclei**. This could lead to
  - Damage of the nuclei (nuclei could lose their RNA content, without any changes in the morphology. This would lead to a failed experiment).
  - Formation of aggregates (nuclei more difficult to be resuspended).

  Optimize spinning conditions in advance.
- **FACS** may be needed/useful to remove debris after nuclei isolation, but given the fragile nature of nuclei, follow these recommendations:
  - RNase inhibitor must be included in collection buffer (account for dilution during sorting).
  - WARNING: FACS should **not** be done **after permeabilization** of nuclei, as they may lose a lot of mRNA content. If planned anyway, a pilot or RNA content check by RNA extraction/quantification is mandatory.
  - **FACSing guidelines** for nuclei can be found in 10XG protocol CG000375, and general guidelines in CG000720
- 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-). For these tissues it may be worth adding RNase inhibitors at more steps than mentioned in your nuclei isolation protocol.
- If the final **resuspension volume** is going to be low and cell pellet is not visible, add some **Diluted Nuclei Buffer/Resuspension buffer** to the nuclei before the last centrifugation. In this way it is possible to leave medium after the centrifugation (decreasing the risk of aspirating away the nuclei), without impacting on the composition of the final resuspension buffer.

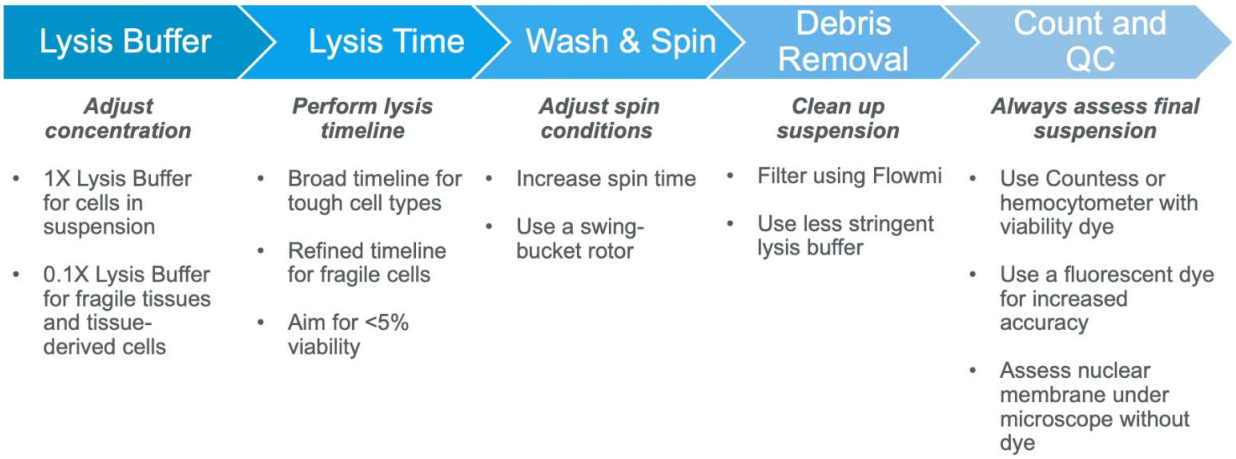
Notes:

- If isolation of nuclei from your tissue fails, refer to this support page: https://kb.10xgenomics.com/hc/en-us/categories/360004142131-Single-Cell-Multiome-ATAC-Gene-Expression
- 10XG support page regarding tissue sample quality and affected metrics can be found here.
- The CryoPrep system from Covaris can be used to isolate nuclei from difficult to dissociate tissues.
- 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, a good starting point to optimize the conditions could be: 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). Optimizing the conditions is important to ensure efficient pelleting while minimizing carryover of debris or aggregation of cells. When doing it the first time, 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 them.
- Frozen samples can be used as a starting material for nuclei isolation, leading to a modest loss of data

quality (see info here). **Nuclei cannot be frozen**. General info about freezing cells can be found here.

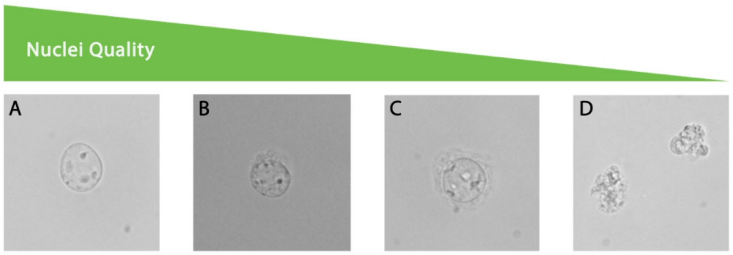
- If it is suspected that the cells suspension contains a lot of ambient DNA, it is possible to treat cells with DNase (at the cells step only, before the nuclei isolation procedure/washes).

Summary slide from 10XG regarding steps that can be tweaked/optimized:



Nuclei quality

- **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 should be **well dissociated** from each other, with minimal amounts of doublets. If performing FACS, carefully gate on FACS to avoid doublets.
- The nuclei prep should contain **no more than 5% alive cells**. These will indeed cluster separately in the scRNA-seq data and complicate the analysis.
- **Avoid cell debris** as much as possible, as they are encapsulated along with nuclei and impact data negatively. If assessing nuclei quality is tricky, a fluorescent stain such as DAPI can be used to differentiate them from debris (but only on the aliquot of nuclei for QC).
- Absolutely **avoid aggregates or clumps** as they may clog the capillaries and lead to run failure. To avoid these, optimize spinning conditions, to avoid "over-spinning". Nuclei can also be passed through a **cell strainer**:
  - Flowmi pipette cell strainer of 40um, cat# BAH136800040-50EA
  - Miltenyi Biotec 30 um PreSeparation Filter, cat# 130041407

- CellTrics filters 20 um, cat# 04-004-2325

- 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.

Nuclei concentration, resuspension buffer type and volume

- The **concentration** of the nuclei must be in the following acceptable ranges, depending on your targeted nuclei number. The last column on the right shows concentrations to aim for:

Targeted Nuclei	*Compatible Nuclei Stock Conc. (nuclei/ul)*	Recommended Nuclei Stock Conc. (nuclei/ul)
500	*160-400*	300-400
1'000	*320-810*	500-800
2'000	*650-1'610*	900-1'300
3'000	*970-2'420*	1'300-1'700
4'000	*1'290-3'230*	1'700-2'100
5'000	*1'610-4'030*	2'000-2'400
6'000	*1'940-4'840*	2'400-2'800
7'000	*2'260-5'650*	2'700-3'100
8'000	*2'580-6'450*	3'000-3'400
9'000	*2'900-7'260*	3'300-3'700
10'000	*3'230-8'060*	3'600-4'000

- Ideally we need **>25ul** of nuclei **at the right concentration** to perform the QC and the run itself in good conditions. If these values cannot be reached, contact us in advance (it is possible to submit down to ca 7-8ul 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 other guidelines do not apply here.

- If FACS is used, consider that **FACS often overestimate cells/nuclei concentration** 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.

- **Nuclei must be brought in the Diluted Nuclei Buffer** that we will give you on the day of the experiment. CAUTION: to save on RNase inhibitor costs, we will give you enough Diluted Nuclei Buffer to resuspend the nuclei in max 200ul final buffer for each sample, with some significant extra safety margin.

  If this volume is not sufficient for the number of nuclei you have, you 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).

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

- FACS buffer contains 1mM EDTA, so make sure the FACS buffer content in the final cell suspension is minimal.

- Place **nuclei on ice** once prepared.

- If used, **trypsin must be inactivated** after use (serum, BSA…).

Starting cell viability

- **Cell viability must be high** before starting the nuclei isolation. **With nuclei, it is difficult to exclude dying/dead cells bioinformatically** as this relies on mitochondrial RNAs, which are absent from nuclei.
- **The acceptable percentage of dead cells** 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, 20-25% 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. Or, measure it on your side.
- **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 (only **7AAD** is accepted).
- Miltenyi offer a **kit to remove dead cells** ("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

- **Multiplexing** solutions are not compatible with multiome, even in an unsupported fashion.
- 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. Avoid introducing batch effects by doing that though.
- 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 well in advance.
- **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, investigate with us if relevant.
- If many **granulocytes** are present, it is recommended to sort them out as they may perform netosis which affects ATAC-seq data quality (see here). Anyway, **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.

- For the **bioinformatics** analysis, when an **ectopically expressed gene** must be added to the reference genome, give us its sequence in advance - including the 3'UTR since the reads will likely map there rather than in the coding sequence. If the transgene is expressed from a lentivector, the reads may lie in the 3'UTR that lies far away in the viral 3'LTR. Similarly, when tags/markers are inserted in 3'/C-ter of endogenous ORFs, reads may lie within these added parts rather than within the endogenous gene. Finally, if the extra gene is already present in the genome, extra care is needed since multimapped reads are discarded by CellRanger.
- **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**.
- 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).

Disclaimer

10XG runs work in general well, but should still be considered at the edge of current technological capacities. There are indeed a non-null 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 nuclei. 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, freeze them as a backup.

Versions log

- vA.01-vA.06: Clarified the volumes of nuclei resuspension that will be given to users, and nuclei number and concentration. Higher safety margin is needed for multiome for targeted cell numbers as compared to applications. Bring unlysed cells to assess viability. Calculations/loading for multiome are very different than for 3'/5'GE and therefore guidelines from these applications cannot be transposed without our approval. For nuclei isolation from complex samples, the optimization is only relative to NP40 lysis buffer and not to the nuclei permeabilization. We load at max 5 ul of nuclei. Mentioned 10XG Chromium Nuclei Isolation kit.
- vA.07-vA.09: major structure change. Clarified that multiplexing is not possible with multiome
- vA.10: Added "without DNA stain" in summary at beginning. Other minor edits.
- vA.11 (26.09.2024): Clarified that if an extra sequence must be added to the reference genome, if it contains an endogenous gene part, reads will be discarded due to multimapping. Added FACS guidelines and a warning regarding FACS on permeabilized nuclei. Mentioned that for RNase-rich tissues, adding RNase inhibitor at more steps may be needed. Encouraged not to push the method too close to the 10k nuclei/sample limit.
- vA.12 (07.10.2024): Added RNase inhibitor concentration to use. Added details about nuclei isolation protocols. Added a disclaimer.