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Multi-Seq: my notes from the lab

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Other

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Human Cell Atlas Method Development Community



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ABSTRACT

Please note these are just my notes and by no means are ground truth of how Multi-seq needs to be performed. I am most WELCOME to comments, suggestions and amendments.

GUIDELINES

Notes are for is for nuclei/CMO; however, for cells/LMO the protocol is the basically the same but using 0.04% BSA because BSA sequesters LMOs (See Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019). You may scale up or down LMO/CMO concentrations while maintaining the same ratio LMO/CMO per cell/nuclei used in paper [that's 8 umol/100K or 40 umol/500K, in a volume of 200 uL, which gives you 40 nM (100K cells) and 200 nM (500K cell), respectively].

MATERIALS TEXT

Materials are those in Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019. You will also need either DNA binding dyes like DAPI, 7-AAD or DRAQ-7 if you are using FANS. For sucrose gradient use this NUC201-1KT (Sigma).

SAFETY WARNINGS

Every below step was done 4°C.

BEFORE STARTING

-I strongly recommend to read Multi-Seq paper, McGinnis *et al.*, Nat Med, 2019.

-Multi-seq is a great tech and not difficult, and yet it needs a bit optimisation to get it to work consistently. Chris and Dave is amazingly helpful blokes and always keen to help if you have any questions. Don't hesitate in getting in touch...I did, a lot!

-Be mindful these are just my notes and by no means are ground truth of how Multi-seq needs to be performed. I am most WELCOME to comments, suggestions and amendments.

- 1 First thing to do is to get **very clean nuclei preps**. You may start with less clean nuclei if you like but it is likely that CMO will bind to the debris, and that is not good (just a thought). So, if you work with cell lines, iPSCs, PBMCs or some of the 'liquid' cancers then nuclei will be pretty clean, but depending on the solid tissues, as you probably have experienced already, you may end up getting dirty nuclei preps.

- 2 To get clean nuclei you can use either a sucrose gradient or FANS based on DNA content staining DAPI/7-AAD/DRAQ-7^(*). I prefer FANS (but this is just me and because I have the sorter in the lab), and for most snRNA-Seq experiments I use DAPI or 7-AAD or DRAQ-7 (I used DAPI more often). After FANS nuclei usually looks super clean and start from there. Sucrose gradient works also quite well, specially to remove myelin from brain tissue, but haven't used it regularly enough. In any case, starting with clean nuclei (when possible) is good practice to try to have the best and reproducible labelling as possible.
(*) These are also dyes are known to not distort chromatin (or very little, as opposed to PI which it does) so we keep this door open for when we do snRNA and snATAC from same sample.
Note: FANS based on DNA content is not just an expensive way to clean up nuclei, it can help you identify potential sub-population with different ploidy, in particular for cancer samples.
- 3 It is important that you visually inspect the nuclei preps under the optical microscope (brightfield) before and after sorting. Check for signs of nuclei damage or partially lysed cells. Nuclei sizes and shape vary a lot, so I don't take this too much into account. I do look at the edges of the nuclei - usually intact nuclei have clear edges. Damaged nuclei are easy to identify with 40x-60x magnification. Sometimes disorganised chromatin popping our out of the nucleus is visible, which is bad sign. Nuclei blebbing is also bad sign.
- 4 Mix Anchor and Barcoded oligos in 1:1 molar ratio in PBS (no Ca²⁺/Mg²⁺) at **0.8 Micromolar (μM)** ^(*), that is 10 times solution (of the duplex) and incubate at **Room temperature** for **00:05:00**. We have been preparing this mix fresh. (*) The working concentration is optional.

What (I think) it's key is to maintain the ratio 40 μmol of CMOs (Anchor-Barcoded Oligo and Co-Anchor) per 500,000 cells (**200 Nanomolar (nM)** final in **200 μl**). So for 5x less nuclei, namely 100,000 nuclei, we use **8 μmol** CMO, which is 5x less.
- 5 The number of nuclei you use of of your choice, for example ~100,000 nuclei^(*) in Washing Buffer [PBS (no Ca²⁺/Mg²⁺) + **1 %** BSA (MACS BSA Stock Solution from Miltenyi, Cat. 130-091-376)]. Note: remember that for cells you use **0.04 %** BSA or no BSA in your Washing Buffer.
If FANS is not an option, then directly resuspend nuclei in 180 μL Washing Buffer and continue with Step 7 below.
We usually chose FANS for our nuclei preps clean up step prior to snRNA-Seq (see *Frankenstein* protocol for snRNAseq using fresh and frozen samples on 10x Platform). (*) Our sorter offset is ~40%, so we sort ~140,000 events to get ~100,000 nuclei.
The sorting is done in **50 μl** of Washing Buffer in 96-well plate (culture plate, round bottom, but LoBind **1.5 ml** eppis work too). I like culture plates because I always inspect nuclei under the microscope after sorting, so these are ideal.
- 6 After sorting, transfer the nuclei to a **1.5 ml** LoBind eppis tube and complete with chilled Wash Buffer to ~ **180 μl** **1.5 ml** (Note: when limited nuclei number, I usually use the additional volume to 'wash' the well where the nuclei come from and then transfer this wash the respective nuclei in the eppi).
- 7 Add **8 μmol** Anchor-Barcode oligo (**8 μmol** = **10 μl** of **0.8 Micromolar (μM)**) and gently but thoroughly mix by pipetting using wide-bore tip. So far we've maintained the **40 μmol** CMO/500,000 nuclei (**8 μmol** CMO/100,000, that's 5x less). According to the authors, for 500,000 nuclei they add **20 μl** of **2 Micromolar (μM)** CMO for a final concentration of **200 Nanomolar (nM)** in **200 μl** final volume).

Here I am accounting for 5x less cells, so I add **10 μl** of **0.8 Micromolar (μM)** CMO in a final volume of ~ **190 μl** (Note: at this stage the Anchor-Barcoded oligo concentration is **42.105 Nanomolar (nM)** , but it'll become **40 Nanomolar (nM)** when I add the Co-Anchor below (bringing the final volume from **190 μl** to **200 μl**). In any case, for 100,000 nuclei Anchor-Barcoded Oligo and Co-Anchor will at the same molarity in the end, that is **40 Nanomolar (nM)** or **8 μmol** each in **200 μl** final volume, which is 5x less of what's used for 500,000 nuclei, namely **200 Nanomolar (nM)**).

- 8 Incubate for 🕒00:05:00 at 🌡4 °C .
- 9 Add 🧴8 umol Co-Anchor and gently but thoroughly mix.
- 10 Incubate for 🕒00:05:00 at 🌡4 °C .
- 11 Add 🧴1 ml of chilled Wash Buffer and mix by inverting 3x.
- 12 🌀500 x g, 4°C 00:05:00
- 13 Remove supernatant without disturbing the pellet. For this, I always leave behind ~ 🧴20 µl - 🧴30 µl of supernatant. This is key to avoid losing the nuclei.
- 14 Repeat wash for 2 more times using 🧴1.2 ml of chilled Wash Buffer. **Do not resuspend** nuclei after every wash – this help reducing nuclei loss.
- 15 In the final wash resuspend in 🧴80 µl of chilled Wash Buffer supplemented with [M]0.2 U/µl - [M]0.5 U/µl of RNase Inhibitor (Protector RNase Inhibitor, Cat. RNAINH-RO, Merck/Roche). One may use RNase inhibitor all the way, but it becomes expensive. I use RNase Inhibitor during cell lysis, and in this final resuspension and RNA/cDNA quality looks good.
- 16 If possible, count using Countess II Automated Cell Counter using Trypan Blue (tend to precipitate so it's a pain. I spin top speed every time I use) or Ethidium Homodimer. Alternatively, proceed with pooling.
- 17 Combine nuclei from multiple samples to multiplex and filter with a 40-um Flowmi® Cell Strainer.
- 18 Count using Countess II Automated Cell Counter.
- 19 Adjust nuclei concentration if needed.
- 20 Proceed with 10x 3' v3 protocol.



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