Chapter 1

Guidance on Processing the 10x Genomics Single Cell Gene Expression Assay

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Abstract

The demand for technologies that allow the study of gene expression at single cell resolution continues to increase. One such assay was launched in 2016 by the US-based company 10x Genomics Inc. Utilizing the power of the single cell on a large scale (Zheng et al. Nat Commun 8:14049, 2017)—capturing thousands of cells at once—has shaped life sciences ever since and allowed researchers to discover new insights within their respective fields of study such as oncology, neurobiology, and immunology (among others). Obtaining high-data quality is the key to being able to make these meaningful discoveries, which in turn is directly linked to the quality of the initial cell (or nuclei) suspension that is used to load the 10x Genomics Chromium Single Cell Gene Expression assay. A successful workflow relies on a cell suspension which is fully dissociated, extremely clean, and of high viability. While the workflow itself has been detailed elsewhere (De Simone et al. Methods Mol Biol 1979:87–110, 2019), in this chapter we will focus on the importance of the quality of the initial cell suspension, as well as common mistakes that can occur while running a Single Cell Gene Expression assay. The descriptions of these tips and tricks refer to the current version of the 10x Genomics User Guide (Chromium Single Cell 3' Reagent Kits User Guide (v3.1 Chemistry Dual Index). https://support10xgenomicscom/single-cell-gene-expression/index/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry-dual-index) which can be downloaded from the Support section on the 10x Genomics website (10x Genomics website. https://www10xgenomicscom). These documents and user guides are continuously improved and updated; hence, it is important to regularly check the company's website for the most recent version.

Key words 10x Genomics, Chromium, Single Cell Gene Expression, Gel Bead-in-emulsion, Cell Ranger, Single cell RNA sequencing, Single cell transcriptomics

1 Introduction

The Chromium Single Cell Gene Expression solution [3] leverages a scalable microfluidic platform to allow for the direct measurement of 3′ digital gene expression at the single cell level. Profiling can be performed for 500–10,000 individual cells per sample, and the resulting data analysis can be used to quantify cell population heterogeneity, characterize cell types and states, and identify dynamic cellular transitions. A pool of ~3,500,000 10x Barcodes

Raffaele A. Calogero and Vladimir Benes (eds.), *Single Cell Transcriptomics: Methods and Protocols*,
Methods in Molecular Biology, vol. 2584, https://doi.org/10.1007/978-1-0716-2756-3_1,

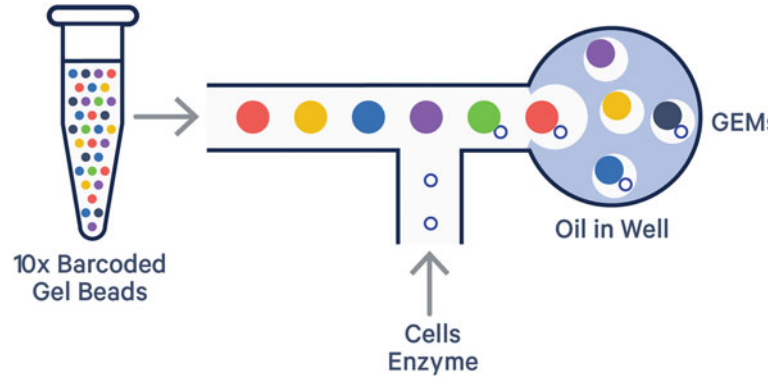

Fig. 1 Gel Beads-in-emulsion (GEM) generation. GEMs are generated by combining barcoded Single Cell 3′ v3.1 Gel Beads, Partitioning Oil, and a master mix containing cells, oligos, and enzymes onto Chromium Next GEM Chip

are sampled separately to index each cell's transcriptome. It is done by partitioning thousands of cells into nanoliter-scale Gel Beads-in-emulsion (GEMs). GEMs are generated by combining barcoded Single Cell 3′ v3.1 Gel Beads, Partitioning Oil, and a master mix containing cells, oligos, and enzymes onto Chromium Next GEM Chip G (Fig. 1). To achieve single cell resolution, cells are delivered at a limiting dilution, such that the majority (~90–99%) of generated GEMs contain no cell, while the remainder largely contain a single cell.

Immediately following GEM generation, the Gel Bead is dissolved, primers are released, and any co-partitioned cell is lysed. Primers containing an Illumina TruSeq Read 1 (read 1 sequencing primer), 16 nt 10x Barcode, 12 nt unique molecular identifier (UMI), and a 30 nt poly(dT) sequence are mixed with the cell lysate and a master mix containing reverse transcription (RT) reagents. Incubation of the GEMs produces barcoded, full-length cDNA from polyadenylated mRNA (Fig. 2). This assay was developed based on human and mouse cell types but has been used for a wide range of various species in the meantime. However, the lysis buffer is not capable of breaking down plant cell walls, which means those sample types require additional optimization upstream of the assay.

After incubation, GEMs are broken and pooled fractions are recovered. Silane magnetic beads are used to purify the first-strand cDNA from the post-GEM-RT reaction mixture, which includes leftover biochemical reagents and primers. Barcoded, full-length cDNA is amplified via PCR to generate sufficient mass for library construction. Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, i7, and i5 sample indexes, and TruSeq Read 2 (read 2 primer sequence) are added via End Repair, A-tailing, Adaptor Ligation, and PCR (Fig. 3).

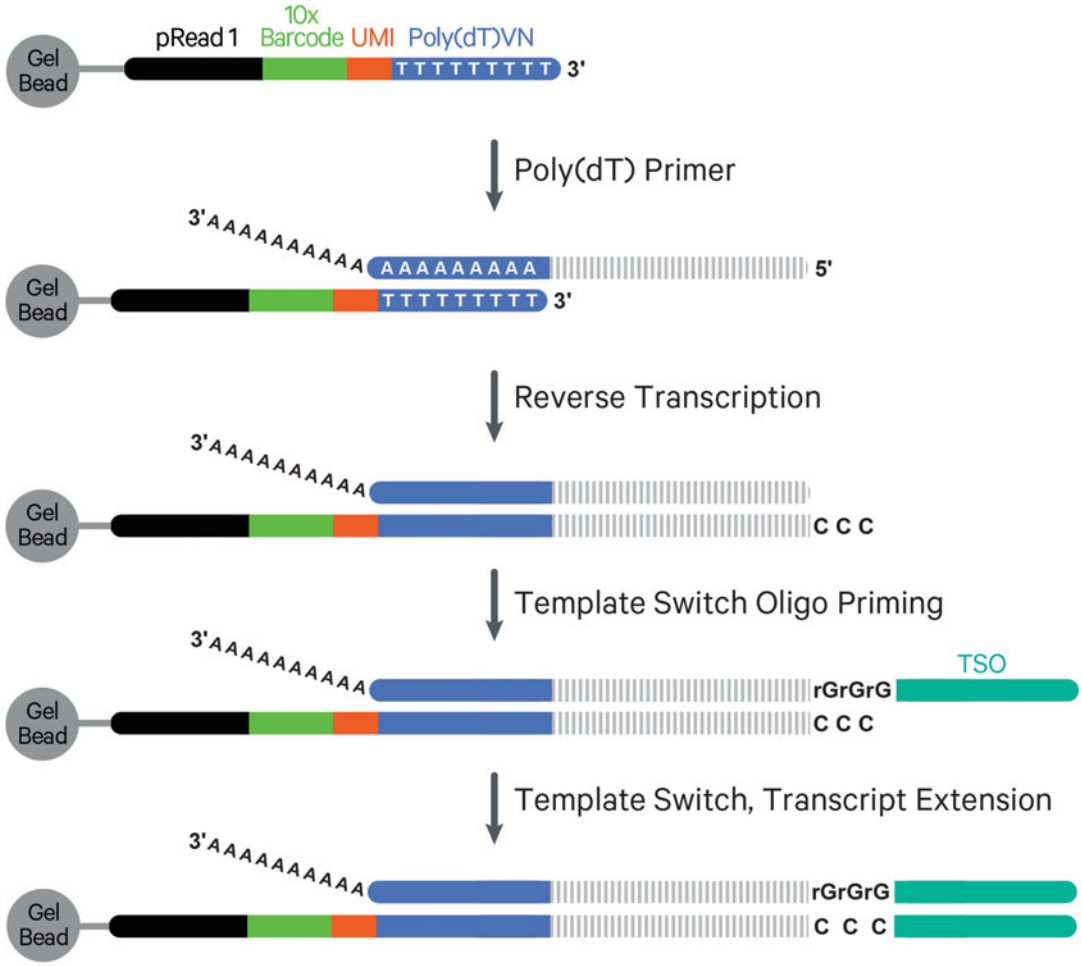

Fig. 2 cDNA generation. Primers containing an Illumina TruSeq Read 1, 16 nt 10x Barcode, 12 nt unique molecular identifier (UMI), and a 30 nt poly(dT) sequence are mixed with the cell lysate and a master mix containing reverse transcription (RT) reagents to produce barcoded, full-length cDNA from polyadenylated mRNA

A Chromium Single Cell 3' Gene Expression Dual Index library comprises standard Illumina paired-end constructs which begin and end with P5 and P7 (Fig. 4). The 16 bp 10x Barcode and 12 bp UMI are encoded in Read 1, while Read 2 is used to sequence the cDNA fragment. i7 and i5 index sequences are incorporated as the sample index reads. TruSeq Read 1 and TruSeq Read 2 are standard Illumina sequencing primer sites used in paired-end sequencing. The 10x Barcodes are used to associate individual reads back to the individual GEMs.

Fig. 3 Libraries generation. Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. P5, P7, i7, and i5 sample indexes and TruSeq Read 2 are added via End Repair, A-tailing, Adaptor Ligation, and PCR

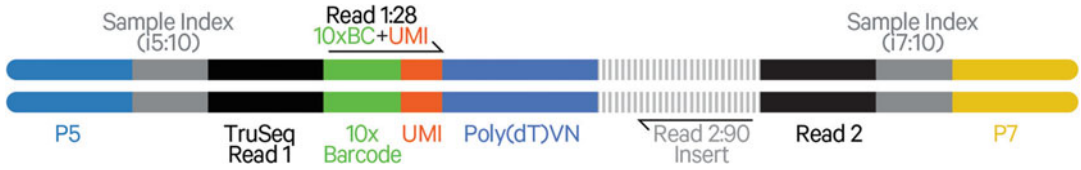

Fig. 4 Chromium Dual Index library. Chromium Single Cell 3' Gene Expression Dual Index library comprises standard Illumina paired-end constructs which begin and end with P5 and P7

2 Materials

2.1 *Plasticware*

It is very important to use the recommended plasticware as indicated in the user guide [3], especially when working with GEMs. This includes the 0.2 ml 8-tube PCR strips and pipette tips. The recommended plastics have been tested and validated to be safe for the emulsions. In rare cases, other plasticware could break the emulsion before the reverse transcription and transcript barcoding

can take place. This would lead to a loss of single cell characteristics for the sample, as the transcripts from the cells and the barcoded oligos would not be confined to an individual GEM droplet, but would float freely within the strip tube in a randomized manner. While the usage of strip tubes is limited to those that are specifically listed in the user guide, and it is strongly recommended to use the brand of pipette tips as indicated in the user guide as well, there are many users who work with alternative pipette vendors without issues, as long as the pipette tips are filtered and low binding (or low retention). Using nonrecommended pipette tips does bear some risks though, as some instances of broken emulsions are not always visible to the naked eye. The usage of DNA LoBind 1.5 ml or 2.0 ml microcentrifuge tubes is recommended to minimize the loss of sample and reagents due to adherence on the walls of the tubes. The same principle applies to the recommended low binding / low retention pipette tips.

2.2 Kits and Reagents

Regarding additional reagents that are required to run this assay, two in particular should be given careful consideration. First, the TE buffer used to resuspend the Template Switch Oligo (TSO) into a working stock solution needs to be of low EDTA concentration, i.e., only 0.1 mM EDTA. This reagent is going to be added to the reverse transcription master mix where the presence of too much EDTA could cause an inhibition of the RT enzyme, thereby causing the experiment to fail. It is recommended to purchase premade low-EDTA TE buffer to avoid any mistakes in final EDTA concentration.

The second reagent requiring special consideration is the SPRI-select Reagent Kit. While it is technically possible to use AMPure XP Beads instead, the use of the SPRI-select Reagent Kit is recommended due to the additional quality control performed by the manufacturer which negates the need to calibrate the beads manually in between different LOT numbers.

2.3 Equipment

It is not strictly necessary to use one of the thermal cyclers listed in the user guide [3]. Many other machines are also compatible with this assay. The important thing to keep in mind is that the thermal cycler needs to have a deep well module which fits 0.2 ml strip tubes and can cover 100 µl of liquid by the module wells. To ensure even heating of the entire 100 µl reaction volume, the reactions inside the strip tubes must not stand over the edge of the wells. Furthermore, the thermal cycler also needs to have a temperature-controlled lid which is capable of holding low temperatures as well. Please refer to the thermal cycler programs in the 10x Genomics User Guide to check on the required lid temperatures. Not conforming to the listed dedicated lid temperatures may compromise assay performance, particularly at the GEM-RT step, as higher lid temperatures could compromise the emulsions. Some thermal

cyclers immediately ramp up to a standard lid temperature upon switching it on; in those instances, ensure that the machine has ample time to equilibrate to the right lid temperature before running the machine with the samples.

3 Methods

3.1 Cell Preparation

Preparation of a high-quality single cell suspension is the key to performing the Single Cell Gene Expression assay and yielding high-quality data from the experiment. This means that the single cell suspension needs to be fully dissociated, clean from various contaminants, and have high cell viability. Technically, this is not part of the single cell assay itself, but it is the most important preparation work for the assay. The extraction, dissociation, and purification of the cells and/or nuclei must be practiced and optimized before attempting to run the actual single cell assay. Some sample or tissue types are much more difficult to work with and will require substantial optimization up front. In the following paragraphs, we will dive into more specifics on how the quality of the single cell suspension should be controlled.

3.1.1 Cell Viability

The viability of the cell suspension should be as high as possible, ideally above 90%, but at least 70%. A high fraction of nonviable or dying cells may decrease cell recovery (*See* **Note 1**).

Cell viability can be checked in several ways. The most common method is Trypan blue staining which will stain dead cells dark blue, while leaving live cells unstained (*See* **Note 2**). Any cell viability below 70% is entering a gray zone for the resulting data quality. The lower the viability drops, the more ambient RNA will be included in the suspension. This ambient RNA could then be captured together with the viable cells, hence increasing background noise. If cell viability is far below 70%, a dead cell removal method could improve the overall cell viability as well as the data quality in the end [5] (*See* **Note 3**).

3.1.2 Cell Counting

When it comes to counting the number of cells in a suspension, manual methods (e.g., a hemocytometer or Neubauer chamber) or automated cell counters are appropriate. Each method has its advantages and disadvantages (*See* **Note 4**). Counting should always be done on the whole suspension, including both dead and alive cells (*See* **Note 5**). Cell counting can be done at various steps during the cell preparation protocol in order to have a better estimate of cell number so that they can be resuspended in an appropriate volume. However, it is important to have an accurate cell count of the final cell suspension that is going to be loaded into the single cell assay (*See* **Note 6**).

3.1.3 Fully Dissociated Cell Suspension

Always inspect the cell suspension under a microscope to ensure it is clean and fully dissociated (*See* **Note 7**).

3.1.4 Clean Cell Suspension

The single cell suspension must be free of aggregates, dust, debris, DNAse, RNAse, enzyme inhibitors, surfactants, and generally viscous reagents [6]. The reasons for these various exclusions are explained in detail in **Note 8**. However, in short, the presence of any of these factors in the single cell suspension could lead to a clog or wetting failure during the chip run on the Chromium instrument, or to a failure to barcode the mRNA from the cells.

3.1.5 How to Keep the Cells Alive and Happy

The default buffer used for cell washing and resuspension is typically magnesium- and calcium-free 1× PBS with 0.04% nonacetylated BSA. However, not all cell types do well in this type of buffer and may require an alternative to remain viable and aggregate-free in suspension (*See* **Note 9**).

The BSA content can also be increased up to 2% to help with cells or nuclei that are sticky and tend to form clumps. Serum up to 10% can be included in the washing and resuspension buffer if it helps the cells, but it is not necessary for the single cell assay itself (*See* **Note 10**). In addition to careful consideration of the chemical properties of buffers used for washing and resuspending cells, there are also physical properties that might need to be checked and optimized in order to obtain a viable single cell suspension: pipetting speed, wide-bore (wide-orifice) pipette tips, use of FACS sorter, temperature, and centrifugation (*See* **Note 11**).

3.1.6 Limited Cell Numbers

When it is necessary to work with very low cell numbers, it is likely that no pellet will be visible after spinning them down. While these situations are more challenging, many researchers face this scenario fairly often. There are a couple of points (listed in **Note 12**) to keep in mind when working with low cell input to get the most out of these samples [7].

3.1.7 Using Nuclei as Suspension Input

The Single Cell Gene Expression assay from 10x Genomics also allows the use of nuclei as input material, rather than whole cells [7, 8]. This can be useful when working with cell types that are too big to fit through the channels of the Next GEM chips. It can also be considered when the dissociation of the cells is too difficult or when tissue has been frozen and the extraction of viable whole cells from such circumstances is not possible (*See* **Note 13**).

3.2 GEM Generation and Barcoding

After ensuring that the single cell (or single nucleus) suspension is of high quality and viability, it can be used as input for the Single Cell Gene Expression assay. The first step is to create a master mix for the downstream reverse transcription (RT) and combine that with the cell suspension (*See* **Note 14**). Next, the final master mix will be loaded into the Next GEM Chip together with barcoded gel

beads and partitioning oil (*See* **Note 15**). Running the chip on the Chromium instrument will generate an emulsion whereby one individual gel bead will be partitioned together with a single cell surrounded by the RT master mix. These GEMs (Gel Beads-in-emulsion) are then transferred to a strip tube, and the reverse transcription reaction will take place on a thermal cycler (*See* **Note 16**).

3.3 Post-GEM-RT Cleanup and cDNA Amplification

At this point, the reverse transcription reaction will have generated the first-strand cDNA from the captured mRNA transcripts, and the resulting RT products carry unique barcodes to their cells of origin. The emulsions can now be broken and the rest of the assay performed in the standard bulk fashion of Illumina library preparation. The barcoded cDNA will have been recovered from the broken emulsions and cleaned using a Dynabead cleanup step (*See* **Note 17**). Now the Read 1 adapter and the attached Template Switch Oligo can be used as primer sites to amplify all of the cDNA products that were captured in the RT step.

Depending on the number of cells targeted for recovery, the amount of PCR cycles used for amplification will need to be adapted (*See* **Note 18**). If nuclei were used as assay input instead of whole cells, it will be necessary to increase the number of PCR cycles by 1–2 cycles during cDNA amplification (*See* **Note 19**). After the amplification, the sample will be cleaned again. From this point forward, SPRI-select magnetic beads will be used for all cleaning steps. When checking cDNA traces on a Bioanalyzer, the user guide [3] recommends performing a 1:10 dilution for loading onto the Bioanalyzer chip (*See* **Note 20**).

3.4 Libraries Preparation and Sequencing

After cDNA amplification, the generated amplicons need to be optimized to be compatible with downstream sequencing (Fig. 3). Enzymatic fragmentation (*See* **Note 21**), which reduces the average size of the amplified fragments dramatically, is followed by an end repair and A-tailing step. Then, a Read 2 adapter can be ligated to the A-tailed ends of the fragments, which will allow attachment of the i5 and i7 sample indices, as well as the P5 and P7 adapter sequences in the final PCR step of the Single Cell Gene Expression assay (*See* **Note 22**). When checking the library traces on a Bioanalyzer, it is possible that a small amount of primer dimers will remain in the sample (*See* **Note 23**). To quantify the final library, a qPCR method, such as the KAPA Library Quantification Kit [9] is recommended (*See* **Note 24**).

3.5 Bioinformatic and Quality Control of the Data

10x Genomics has a license-free Linux pipeline called Cell Ranger that can be downloaded from the 10x Genomics support website [10] and used for data analysis of the Chromium Single Cell Gene Expression assay. The details of how this pipeline functions are beyond the scope of this work, but a description can be found at https://support.10xgenomics.com/. Of greater importance is the

interpretation of the data after it has been processed by Cell Ranger [11–13]. The key output file for this purpose is the web_summary.html file, which contains the quality control metrics for the sample that was processed. There is a Technical Note available that helps with the interpretation of these metrics [14], but the most important ones are summarized in **Note 25**.

4 Notes

1. Premature cell lysis and cell damage release mRNA from dying cells into the microcentrifuge tubes containing the single cell suspension. This ambient mRNA is suspended among the viable single cells and will therefore also be loaded into the microfluidic chip during the master mix preparations. During the Chromium run, the ambient mRNA will be captured inside the emulsion droplets together with viable cells. These ambient transcripts will be barcoded with the same 10x Barcode as the transcripts originating from the viable cell, thereby contaminating the GEM and the data coming from this cell. The ambient mRNA from dead, dying, or lysing cells in the single cell suspension will cause background noise in single cell data and should be avoided at all costs.
2. Trypan blue can build up precipitates over time which are visible as dark blue flakes during counting. This could potentially lead to inaccurate results when using an automated cell counter. In this situation, it might be necessary to filter or spin down the Trypan blue tube before using it for cell staining, so that the solution remains free of precipitate. One alternative to Trypan blue staining is dual-fluorescence with acridine orange (AO) and propidium iodide (PI) viability staining [15]. While PI can only stain dead cells due to their damaged cell membranes, AO can permeate membranes and therefore stain both dead and live cells. In some circumstances, especially with samples containing heterogeneous cell populations, this method can be advantageous over the Trypan blue staining method.
3. Two examples of dead cell removal commonly used methods are as follows: (i) Live/dead fluorescence-activated cell sorting (FACS) of the cell suspension, by using a viability marker to determine plasma membrane integrity. (ii) Dead cell removal, by using the Miltenyi Biotec Dead Cell Removal Kit [16] to magnetically label cell debris, dead cells, and dying cells, which will then be removed by magnetic separation over a column.
4. Automated cell counters are faster and more streamlined than manual counting. Furthermore, (some) automated cell counters will allow the possibility of retaining images of the cell

suspensions, which can be very helpful for troubleshooting purposes. However, some machines could struggle to count accurately when: (i) the cells are extremely small and are falsely recognized as debris or dead cells, (ii) the focus is not set correctly, (iii) the loaded cell density is too dilute or too concentrated [17], (iv) there are many cell aggregates, and (v) there are big differences in cell sizes within heterogeneous cell suspension. These factors could lead to inaccurate cell counts, and some of these concerns also apply to manual counting, of course [18]. The disadvantages of manual cell counting are the extended time it takes to count the cells and the variability in counting outcomes between users based on the individual user experience.

5. Dead cells will still be present in the cell suspension that will be loaded into the chip on the Chromium instrument, and the downstream data analysis pipeline could potentially still recognize those cells. Ideally, cell counting should be performed at least in duplicate for higher accuracy, with the standard deviation of replicate counts being below 25% [19].
6. Filtering the cells with a cell strainer could lead to a loss in cell number and/or cell concentration, depending on which strainer is used. The cell suspension should also be recounted after FACS sorting as the cell numbers tend to be overestimated by FACS machines. It is important to count the suspension that is actually going to be used for the downstream steps. While some researchers like to sort cells into two different wells or tubes and then use one of them for counting and the other for loading the single cell assay, this practice could lead to discrepancies in cell numbers and should be avoided.
7. Cells that are still stuck together in multiplets or larger aggregates are not in single cell suspension. If included in the suspension loaded onto the Chromium instrument, these nondissociated cells will be encapsulated together inside the same GEM and will contribute to multiplets, which will be generated during the single cell assay.
8. *Aggregates, debris, and dust* The channels of the Next GEM chips that are processed on Chromium instruments have a fixed width. The maximum recommended cell diameter is 30 μm (in suspension). Loading cells with diameters up to 40 μm might be possible, but cell recovery rates may be compromised. The microfluidic channels themselves are the limiting factors here. While there is no lower cell size limit, cells that are larger than 40 μm in diameter could potentially clog the channel and lead to a loss of the sample. Furthermore, anything in the suspension that is not a single cell, e.g., cell aggregates, clumps, dust particles, or debris, could lead to the same failure type, if they are larger than the recommended cell diameter. The cell

suspension should be checked under a microscope to ensure there are no contaminants present. This check can also be done while performing the cell counting. If the suspension is not visibly clean, it should be filtered through a cell strainer with a maximum filter pore size of 40 μm to ensure that any particles larger than 40 μm are removed from the suspension. Further information on clogs is covered below, in the Subheading 3.2. Note that high levels of debris can also result in a wetting failure, in which emulsions will not form correctly and the single cell characteristics of the sample will be lost.

DNAses and RNAses The presence of these contaminants in the cell suspension is problematic as they will be loaded and partitioned inside the GEMs together with the cell master mix. Once the cells are lysed and reverse transcription has started, RNAses and DNAses can destroy the mRNA that would otherwise be captured, or destroy the barcoded cDNA intermediate product that would be generated during this step.

Enzyme inhibitors If enzyme inhibitors are part of the single cell suspension, they will be included in the GEMs produced by the Chromium instrument. The very first step of this assay is enzymatic, and if the reverse transcription is blocked by an enzyme inhibitor, the barcoded cDNA will not be generated. Media should not contain excessive amounts of EDTA (>0.1 mM), or magnesium (>3 mM) as those components will inhibit the reverse transcription reaction.

Surfactants and viscous reagents Another possible problem during a chip run is a "wetting failure," a phenomenon that occurs when the emulsion cannot be formed correctly. This topic is covered in more detail in Subheading 3.2. The presence of surfactants or any form of viscous reagents in the single cell suspension could lead to an instability of the emulsion formation. Failure to form GEMs means the cells will lyse in an uncontrolled manner and the primers from the gel beads will be released into the reaction mixture at random as well. In short, a wetting failure leads to the loss of single cell characteristics.

9. When cell viability and/or clumping problems are observed with the default 1× PBS + 0.04% BSA buffer, the buffers listed below can be tried instead. They have been verified to be compatible with 10x Genomics Chromium Single Cell Protocols with little or no loss of performance when profiling [7]: Dulbecco's Phosphate-Buffered Saline (DPBS), Hank's Balanced Salt Solution (HBSS), Eagle's Minimum Essential Medium (EMEM) + 10% FBS, Dulbecco's Modified Eagle

Medium (DMEM) + 10% FBS, Iscove's Modified Eagle Medium (IMEM) + 10% FBS, Roswell Park Memorial Institute (RPMI) + 10% FBS, Ham's F12 + 10% FBS,1:1 DMEM/F12 + 10% FBS, M199.

10. It is important to note here that the list of alternative buffers and media above (*See* **Note 9**) is not exhaustive. Other media might be compatible, but they have not yet been tested. As long as the medium does not contain any of the disruptive ingredients listed in Subheading 3.1.4, they will pose a low risk. This is especially the case if the unknown buffer or medium has been used upstream during the cell dissociation process and has been sufficiently washed out before the final cell suspension is loaded into the actual Single Cell Gene Expression assay.

11. Two factors that are often overlooked at this point are as follows: (i) pipetting speed and (ii) the usage wide-bore (or wide-orifice) pipette tips. (i) Pipetting speed. The speed of pipetting while washing and resuspending cells is a subjective matter. One needs to keep in mind that cells are generally fragile, even the ones deemed to be robust. High-speed pipetting, as would be commonly used for a master mix, can be too harsh for cells. Pushing the cells at high speed through the narrow opening of the pipette tip can create shearing forces on the cells. This will then cause their cell membranes to rupture, releasing their mRNA contents into the microcentrifuge tube, hence contaminating the suspension with background noise through the presence of this ambient RNA. Slower pipetting speeds or the use of wide-bore pipette tips can help to reduce the shearing forces exerted on the cells. (ii) Wide-bore (wide-orifice) pipette tips. Some vendors offer wide-bore pipette tips for P1000 (and sometimes also P200) volumes. Using such ready-made pipette tips is the recommended method for washing and resuspending cells. Cutting normal pipette tips by hand to make wide bore tips would improve the shearing forces due to the wider opening of the tip, but this method causes other problems. By cutting the plastic, sharp edges can be created that would offset the reduced shearing forces. Furthermore, tiny pieces of plastic could break off and fall into the cell suspension, thereby potentially causing a clog during the Next GEM Chip run on the Chromium instrument. Additional physical properties that should be considered when optimizing the cell preparation are FACS, temperature, and centrifugation.

    In order to maintain cell viability and integrity when using a FACS sorter, it is not recommended to sort into dry tubes. Also, it is important to keep in mind that using FACS is not completely risk-free as some sensitive cell types can become

leaky due to the sorting pressure. By default, cell suspensions are kept on ice during cell preparation protocols. However, not all cells do well when kept on ice as the cold temperature could cause them to stress, clump, or die. Some fragile cells might be better maintained at room temperature. The speed and timing of centrifugation might need to be adjusted for the particular cell types that are being studied. The default speeds and times listed in various protocols have usually been optimized for very specific cell types. When working with cell types that are larger or smaller, both the centrifugation speed and duration should be modified as necessary. In general, smaller cells might benefit from faster speeds, while larger or fragile cells might be better suited to slower speeds but longer centrifugation times.

12. *Washing and resuspension buffers.* Use the correct buffer for washing and resuspending the cells. If the cells are coming from a FACS machine, consider sorting them directly into the right buffer and then keeping them in the same type of buffer throughout the downstream processing steps. This way no buffer changes are needed, thus minimizing handling of the sample and loss of cells.

Straining If it is necessary to strain the cells, do so while the cells are still dilute and before spinning them down for the first washing step.

Counting Count the cells before the first washing step to estimate the appropriate buffer volume for resuspension.

Centrifugation Optimize the centrifugation speed and duration for your cell type. Always use low-binding centrifugation tubes to avoid unnecessary adhesion of cells to the tube walls. Depending on the centrifuge and rotor type, use of either 2.0 ml round-bottom or 1.5 ml V-shaped bottom microcentrifugation tubes can help with pellet placement. V-shaped tubes in a swing-bucket rotor centrifuge will place the pellet in the tip of the tube, allowing for very precise localization of the cell pellet, even if it is not visible to the eye.

Washing Remove the supernatant very carefully, recovering as much as possible, but retain the supernatant until after the washed and resuspended cell suspension has been checked. When the cell numbers are low and the centrifugation conditions are not entirely optimized yet, it is possible to accidentally take up the cell pellet while washing. Retaining the supernatant makes it possible to go back to it if cells are found to be missing during the final cell counting. Sometimes even careful pipetting of the supernatant is going to lead to the accidental removal of cells. If this continues to happen, consider leaving a bit of the supernatant behind (~50 μl) and resuspending the cells within the remainder of the supernatant.

This method will clean the suspension as well as the complete removal of the supernatant, but in some rare cases it might be the best option.

13. When the nuclei originate from a cell suspension, it is important to ensure that the stock cell suspension has high viability, ideally above 90%. Even though the cells will eventually be lysed, initial cell viability is directly related to the quality of the nuclei themselves. If the viability of the cell suspension is low to begin with, nuclei quality will suffer as well. In order to find the optimum duration for lysis, perform a lysis time course. Check cell viability at the beginning of the lysis as a reference point and then continue to check on cell viability regularly throughout the timeline. Ideally, there should be between 1 and 5% live cells remaining after a successful lysis. This ensures that the cells have not been overly lysed, which could happen if cell viability after lysis is 0%. Another quality control check for nuclei is to assess their membrane integrity under a microscope at 60× magnification. This should be done with and without staining solution. The membrane is more easily defined when it is unstained, and staining also makes the nuclei look swollen and sometimes grainy, depending on the nuclei type. First, look at the nuclei at low resolution to gauge the level of clumping. This can be done on a cell counter while counting. If there is extensive clumping, this could be a sign of potential over-lysis. After confirming that the nuclei suspension is clean, focus in at 60× magnification and look at a good number of nuclei to gauge membrane integrity. It is important not to cherry pick only the best-looking nuclei membranes. Counting nuclei suspensions can be tricky, especially if there is a lot of debris present from cell or tissue dissociation. In that case, using a fluorescent method such as ethidium homodimer-1 or other fluorescent dyes is advised.
14. It is recommended to make the master mix fresh and only after the final sample number has been confirmed. During sample preparation, it may become necessary to exclude a sample from the assay for various reasons (e.g., when cell viability is too low). In this case, preparing the master mix in advance would waste reagents since volumes would be calculated to accommodate a higher number of samples than will actually be processed. Before resuspending the Template Switch Oligo (TSO) with low-EDTA TE buffer, confirm the location of the TSO pellet. During transportation, it may have come loose and could be stuck in the cap of the tube. Always spin down the tube before opening it. The remaining volume of resuspended TSO must be stored at −80 °C to ensure the proper efficiency of the primer. Placing the TSO tube inside the box with the Gel Beads is a convenient solution that prevents the tube from

getting lost in the −80 °C freezer. When preparing the master mix, ensure that the correct volumes of the cell suspension and nuclease-free water are used. There are two options for determining these volumes: (i) Following the user guide. To help with the concentration calculations of both cell suspension and water, a pipetting table is included in the Single Cell Gene Expression user guide [3]. If the cell concentration of the stock solution and the number of cells targeted for recovery are known, the table can be used to determine the exact volumes of water and cell suspension required, as the cell recovery efficiency of ~60% is already built into that table. Important: Do not mix the cells with water! The table is an overview of the volumes required. Once you have generated the master mix and aliquoted it into the strip tubes, add the appropriate amount of water to the master mix and combine thoroughly. Into this master mix and water combination, add the appropriate volume of cell suspension. Use a P200 pipette tip to pipette up and down 5 times, with both the aspiration and dispersion steps each taking 3 s to complete. Using a multichannel pipette at this point is recommended. Once the cells are fully combined with the master mix, use the same pipette tip to transfer the suspension into the Next GEM Chip. Using the same pipette tip to mix and transfer avoids sample loss that could occur by unnecessarily switching to fresh pipette tips. (ii) Deviating from the user guide [3]. Some researchers prefer to calculate their own volumes for cell suspension and water. Instead of referring to the pipetting table before each run, they always load a fixed volume of cell suspension. This way, they also have a fixed volume of water which can be directly added into the master mix before it is aliquoted into the strip tubes. The desired cell concentration is calculated beforehand and adjusted within the fixed volume of cell suspension.

15. When loading a Next GEM Chip, there are several aspects that need to be kept in mind. The chip is an open system with microfluidic channels. In order for the Chromium instrument to process the liquids on the chip correctly and successfully form single cell emulsions, it is important to be aware of the potential reasons that a run might fail.

Clogs A clog is a situation where the emulsion was able to form correctly, but one of the channels became blocked and the remainder of the reagents was not able to flow through. The emulsion will look normal in appearance, but it will have less volume than it should. An example of this can be seen in pipette tip C in Fig. 5.

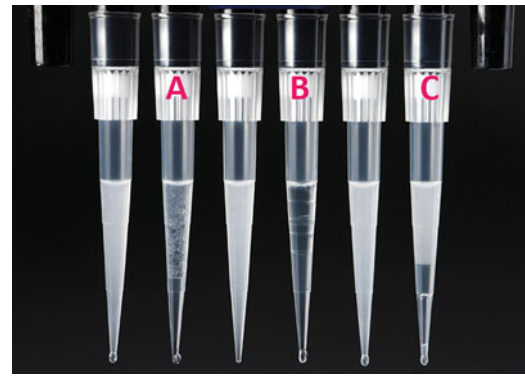

Fig. 5 Critical issues in the emulsion chip processing. (**a**, **b**) Images of incorrect emulsion formation that cause wetting failure; (**c**) example of clog which prevents reagents to flow through the chip

To avoid clogs, it is recommended to strain the cell suspension with a cell strainer that has a maximum filter size of 40 μm. Using a cell strainer with larger filter pores could potentially still include particles that are too big for the chip channels. However, the most overlooked risk factor is probably dust. Dust is always present in labs, and no matter how clean the cell suspension is, if a dust particle ends up in one of the channels of the Next GEM Chip, it can still cause a clog. To minimize this risk, cover every component that will eventually be loaded into the chip in some way, unless it is actively being used. This includes the chip itself, the individual reagent tubes from the Single Cell Gene Expression kit, the microcentrifuge tube used for preparing the master mix, the microcentrifuge tubes containing the cell suspensions, and the strip tubes used for the master mix and cell suspension aliquots.

Wetting failures A wetting failure means that the emulsion was not able to be formed correctly. In this case, cells will lyse and the Gel Beads will dissolve freely, without being confined to an individual emulsion droplet. This means that the sample will lose its single cell characteristics. Examples of wetting failures can be seen in pipette tips A and B in Fig. 5. Please note that there are varying levels of wetting failures and some are easier to spot than others. Always use a dark background (as shown in the figure below) when checking on the emulsion quality after the chip run. The reasons for wetting failures are quite varied [20], but the most common issues are temperature, loading orientation, pipetting speed, priming, pipetting style, air bubbles, and sample quality.

Temperature The Chromium instrument needs to be operated within a specific temperature range of 18–28 °C. The Gel Beads also need at least 30 min to warm up to room temperature (i.e., the same range as required for the Chromium instrument) before use. Placing and running the machine next to a south-facing window is

not recommended as the temperatures will be too high for proper emulsion formation. The same logic applies to cold conditions—do not place and run the Chromium instrument within a cold storage room or in a nonheated room in winter. Operating the instrument outside of these conditions could cause a wetting failure because the emulsions consist of a delicate balance of oil, water, and (strictly controlled) viscous reagents such as Gel Beads.

Loading orientation The wells of the Next GEM Chip are labeled with the order in which they need to be loaded: the cell suspension combined with the RT master mix is loaded into Row 1, the gel beads are loaded into Row 2, and the partitioning oil is loaded into Row 3. Since the chip is an open system, as soon as liquids are loaded into the wells, they will be pulled through the channels by capillary forces. In order for the emulsions to be formed correctly, the reagents inside these channels need to be at the correct place at the correct time. Loading the wells in the correct order ensures this happens. Reagents loaded in reverse order will cause a wetting failure and loss of the sample.

Pipetting speed This is often overlooked in the user guide, but the appropriate speed for pipetting reagents into the chip is discussed in that document. Pipetting needs to be slow, such that dispensation of the reagents into the wells should take 5 s. This will ensure that the liquids are not pushed into the wells and into the channels. Faster pipetting could force the reagents through the channels and their junctions where they would otherwise not flow naturally. The result would be the same as if the reagents had been loaded out of order. *Priming*. There is always a 30 s waiting period between the loading of each row of reagents. This allows the capillary forces to prime the chip by pulling the reagents through the channels where they need to go in order for the emulsions to be formed correctly. *Pipetting style*. Always pipette into the center of the wells (Fig. 6) and slowly raise the pipette tip while dispensing the reagents within the required 5 s time frame.

Special care should be taken with the partitioning oil as this will be a small reagent volume pipetted into a large well. The partitioning oil needs to be in direct contact with the center section of the wells in Row 3, as this is where the gel beads and cells will come together to form the GEMs.

Air bubbles In general, avoid air bubbles as much as possible when loading the chip. However, there are some forms of bubbles which are far more damaging than others. If an air bubble gets stuck at the bottom of the well, it could cause the reagents to flow incorrectly through the channels, thereby creating a wetting failure. After loading all of the wells, carefully tilt the chip to check that no air bubbles are trapped at the bottoms of the wells. If an air bubble is

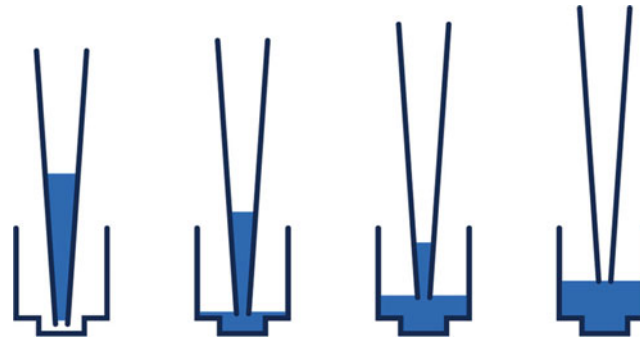

Fig. 6 Correct pipetting mode. Pipetting into the center of the well is strongly recommended

visible, carefully nudge it with a P10 pipette tip to dislodge the bubble. Once the bubble has floated to the top of the well, it is safe to proceed with the chip run. If there are bubbles at the top of the well and they cannot be removed with a P10 pipette tip, they can remain in the wells for the chip run, as they are small enough to not interfere with the assay. However, to be on the safe side, a needle can be used to get rid of even these small bubbles.

Sample quality Wetting failures can also be caused by poor sample quality in general. If suboptimal samples are used (e.g., suspensions that contain a lot of debris or dead cells), the risk of obtaining a wetting failure may increase.

Even when taking all of these precautions, it is still possible for a clog or wetting failure to occur. These failures can be observed at a low, single-digit percentage of the samples being processed. Fortunately, these problems will be apparent immediately after the Chromium instrument has finished its run and the emulsions need to be transferred to the strip tube for further processing on the thermal cycler. Monitor the Chromium instrument during the first minute of each run to ensure that there are no error messages. Next, set a timer to be sure to be back at the Chromium instrument just as the run is finishing. Do not let the chip sit before transferring the GEMs. If you notice a problematic sample that is showing signs of a clog or wetting failure like one of the examples from Fig. 5, there may still be a chance to save the sample. If the sample is precious and the experiment cannot be repeated, prepare a new chip and a new master mix and run the cell suspension again. In most cases, the repeat run will succeed and the assay move forward with all samples. If the issue was a wetting failure and the repeat of the sample is showing this same problem again, the sample will need to be discarded. In that case, a problem with sample quality or something in the cell suspension buffer is causing the emulsion to fail. Trying to repeat the run with the same buffer composition will

not succeed. Secure all samples for proper storage and then return the 10x Genomics reagents to −20 °C or −80 °C.

Sometimes, the exact number of cells in a final cell suspension is unknown because the cell numbers are so low that it is too much of a risk to lose cells through the counting process. In this case, researchers might choose to skip the addition of the water to the master mix and use the full volume of the cell suspension instead. While the cell number will remain unknown, this option aims to capture as many cells as possible. This is a valid approach, however, there are risks involved. Although the odds of a run failure are very low, if one occurs, it would be a considerable loss for a sample that was extremely rare and precious. If working with such precious samples where the total cell suspension volume is too low to repeat if it should fail, one possibility is to run only half of the suspension, no matter the desired number of cells. This means capturing fewer cells, but if something goes wrong during the run and the sample is lost through a clog or a wetting failure, there is still the opportunity to repeat the sample and save it. Researchers will have to weigh the options and decide for themselves the best way to proceed for their given experiment.

16. As soon as a run has been completed on a Chromium instrument, the emulsions need to be recovered from the Next GEM Chip and transferred to a strip tube immediately. The pipetting speed for aspirating the emulsions is specified at (at least) 20 s. The dispensation into the strip tubes should be done at the same speed, for the duration of (at least) 20 s. This is done to avoid breaking the emulsion and also to allow the liquid to leave the pipette tip. The emulsion is slightly viscous, and dispensing it too quickly will leave emulsion behind inside the pipette tip, causing unnecessary sample loss. Using a multichannel pipette for this step is highly recommended. However, if a pipette tip falls off during this step, do not attempt to pipette the emulsions up and down to recover the lost sample. First, finish pipetting the other channels and then use a single channel pipette to recover the sample from the fallen tip. Proceed immediately to the reverse transcription section of the protocol. The emulsion can be stored on ice for up to 1 h if there is a second chip being processed on the Chromium instrument and both sets of samples will be run together on the thermal cycler. Please note that the assay has been developed and validated with a limited number of cell types. While this is a safe practice and can be used for running multiple chips back-to-back, it is possible that this delay in starting reverse transcription might not be suitable for certain cell or sample types. When loading the thermal cycler, place empty tubes on the corners of the block to avoid sample tubes being crushed by the lid, especially when running less than four samples.

Furthermore, ensure that the heated lid option is set at the correct temperature; running the heated lid at high temperatures (e.g., 100 °C) will break the GEMs prematurely. The thermal cycler program can be adjusted with an additional hold step at 53 °C at the beginning of the run. This would ensure that the lid and sample block are already at the correct temperature for the incubation of the GEMs when they are loaded onto the block. If you are incorporating this additional holding step, do not forget to advance the program to the actual RT steps. Forgetting to advance the program to the RT process is far more damaging to the sample than not including the optional preheated holding step. The finished RT reaction can be held at 4 °C overnight in the thermal cycler if scheduling makes it convenient to do so.

17. The emulsions are broken with the help of the pink Recovery Agent. No amplification rounds have been performed at this stage so each cDNA molecule is only represented once. Be careful not to lose material during the cleanup steps as there are no duplicates for any lost transcripts and that data cannot be recovered. After the Recovery Agent has been added, the upper aqueous phase will contain the barcoded cDNA product and the bottom pink phase will need to be discarded. In the user guide [3], the upper phase is shown with a clear, see-through appearance. Under certain circumstances, this upper phase could look differently [21] and may appear milky instead of clear if the cell suspension contains a high concentration of BSA or FCS; appear slightly pink or red if the cells were resuspended in a culture media of such color; and appear cloudy if the GEMs had been frozen at −20 °C after reverse transcription. After the incubation and breaking of the emulsion, be sure to pipette slowly when aspirating the pink Recovery Agent from the bottom of the strip tubes. If everything went well and there were no issues with the run, there will still be a bit of pink fluid left over at the bottom of the strip tubes after removing the indicated volume of Recovery Agent. This is done by design. If while pipetting, pink fluid is running low at the bottom of the well before reaching the top of the aspiration, simply stop earlier than the full volume of the pipette's setting. Continuing to pipette after the pink Recovery Agent is depleted would mean pipetting away and discarding sample. Avoid this at all costs. The Recovery Agent is viscous, so pipette slowly to allow the reagent time to settle down into the bottom of the tube. Once the Recovery Agent is removed, Dynabeads are used for sample cleanup. Before vortexing the Dynabeads, mix the whole volume of the Dynabeads reagent by pipetting up and down, as it is possible for Dynabeads to pellet at the bottom of the tube. Vortexing alone might not always be

efficient enough to dislodge that magnetic bead pellet. Make sure to vortex the tube for at least 30 s and then pipette the fresh magnetic beads directly to your cleanup master mix. The magnetic beads are quite heavy and will settle very quickly. If settling results in an incorrect concentration of magnetic beads in the cleanup master mix, this will lead to less efficient cDNA recovery. When adding the cleanup master mix to the strip tubes, do not attempt to close the lids on the strip tubes. The strip tube is designed for 0.2 ml fill volumes, but they will contain 300 μl at this point in the protocol. The full volume will fit, provided that the lid is kept open. The bottom phase will appear pink rather than white as indicated in the user guide [3]. While mixing, take care to mix the full depth of the tube, including the pink phase at the bottom (i.e., actively pipette into the bottom of the tube and then release the suspension from the top of the tube to maximize the mixing of the cDNA with the magnetic beads). It is normal to mix the pink phase alongside the upper brown phase, but that pink phase will settle down again immediately. A white interphase can sometimes be present between the bottom pink phase and the upper brown phase. This is completely normal and nothing to worry about. Following the incubation period, in which the cDNA is bound to the magnetic beads, place the strip tube on the Magnetic Separator. The magnetic beads will be pulled to the back of the strip tube into a small, tight brown pellet consisting of the magnetic beads with bound cDNA. The initial supernatant recovery calls for the removal of everything from the tube except the magnetic beads. This includes the pink phase and (if present) the white interphase at the bottom of the tube. After that, simply follow the user guide protocol for standard cleanup. Always make sure that the magnetic bead pellet is wet in some way, either with ethanol or the elution solution at the end of the protocol. The indicated air-drying times should not be exceeded, but rather strictly maintained to avoid overdrying of the bead pellet and a corresponding loss of cDNA product. Please note two important aspects of the Magnetic Separator from 10x Genomics:

(i) Magnetic pins may come loose. The tiny round pins at the back of the magnet could move to the outside or even fall out completely because they are not fixed nor glued in place. This is of particular concern if using a magnetic wall for storage. It is not an ideal storage location—when pulling the magnet off the wall, the magnetic pins at the back could be removed from the magnet. Even if the pins are simply moved a bit (instead of falling out entirely), this would lead to a bigger gap between the magnets and the magnetic beads carrying the sample, therefore leading to a worse

recovery of the cDNA. If the magnet has been stored on a magnetic wall, make sure to check on the magnetic pins at the back and push them back into place if necessary.

(ii) Magnets are asymmetrical. There are two markings edged into the Magnetic Separator at the top and bottom of the frame. These indicate the High and Low positions of the magnets, which have been designed to be asymmetrical in the holder. If using the recommended Eppendorf strip tubes, the High and Low positions can be used as guidance, as indicated in the user guide. However, the recommended Microamp strip tubes do not reach the magnetic pins if the tubes are placed into the Low position of the Magnetic Separator. Due to their design, the Microamp strip tubes sit higher inside the magnet, so only the High position of the magnet should be used, and the High/Low indications in the user guide should be ignored. Last but not least, the magnet can always be switched between the High or Low position if the magnetic beads are not pelleting in an optimal way. Use of the High and Low positions as indicated in the user guide is not a firm rule.

18. Low cell numbers require more amplification, while large cell numbers will need less amplification. This means that amplification cycle numbers will vary between sample batches. Therefore, always confirm the PCR programming, and in particular the cycling conditions, when starting the thermal cycler to be sure that the amplification cycle number is appropriate for each set of samples.
19. Nuclei contain only a tiny fraction of the total RNA from the whole cell, so more amplification is needed here than with intact cells.
20. This dilution factor might be too high for some samples during quality control, especially when working with tiny cells or a small number of total cells. Instead, consider doing a 1:3 or 1:4 dilution which may prevent the need to repeat the Bioanalyzer run if the 1:10 dilution turns out to dilute the sample too strongly, beyond the point where traces can be correctly identified. Alternatively, a Qubit run could be used first to check on the concentration, providing information needed to load the Bioanalyzer chip within its correct range of detection. Furthermore, the cleaned, amplified cDNA is in a stable state at this stage and can be stored at −20 °C for up to 4 weeks. Some researchers may prefer to hold here and batch a couple of sample runs together on a Bioanalyzer chip or Tapestation screen tape before checking on the traces.
21. Enzymatic fragmentation is controlled only through temperature and duration, which means everything needs to be

precooled on ice, including the microcentrifuge tube used for the master mix and the strip tube with the samples. Furthermore, it is important to note here that the fragmentation program on the thermal cycler is programmed to start at 4 °C, meaning that the block will be cooled down to 4 °C when loading the samples. However, do not forget to press Start or Skip (depending on the thermal cycler model) to actually advance the fragmentation program through the 4 °C hold stage. Both the beginning and the end of the program hold at 4 °C and upon returning to the machine and seeing the 4 °C readout, it is easy to assume that the run has been completed, when instead it never progressed through the fragmentation. During this phase of the protocol, there are a few double-sided SPRI cleanup steps, in which fragments that are too small and fragments that are too big will be removed from the sample. In a standard SPRI cleanup, the large cDNA fragments are bound to the magnetic beads. The ratio between the sample and the added magnetic beads determines the fragment size that can be captured by the beads. This means that the smaller fragments are left behind in the supernatant which is usually discarded in typical one-sided SPRI cleanup. For a double-sided SPRI cleanup, the supernatant is kept at first, and the magnetic beads are discarded instead. This step removes fragments which are too large, as they are bound to the magnetic beads. Then, magnetic beads are added at a different ratio, and the supernatant is again discarded. This allows for sample cleanup within a very specific fragment size range. The most important point to remember for a double-sided SPRI cleanup is to not throw away the supernatant by force of habit.

22. When setting up the Sample Index PCR, make sure to choose the correct Sample Index plate as there are quite a few different ones available these days, and each one is very specific to both the assay and the library type that can be generated. Also, always write down which index was used for which sample; this information will be needed downstream during the data analysis. The number of PCR cycles needed for the Sample Index PCR depends on the cDNA mass that was calculated during the Bioanalyzer check of the amplified cDNA intermediate product. Keep in mind that the assay continued with only a quarter of the total cDNA mass, so the number of PCR cycles needs to be appropriate for 25% of the total cDNA that was measured. Furthermore, when considering PCR cycle number, one should always follow this simple guideline: amplify as much as is needed, but no more than is necessary.
23. If this is the case, evaluate whether it might be less expensive to increase the sequencing depth to compensate for this, versus

re-cleaning the library or re-constructing a new library from the leftover cDNA intermediate before sequencing the sample.

24. The reason being that this kit uses the P5 and P7 adapter sequences as primer sites, ensuring quantification is based on a complete and correctly built library. If the P5 and/or P7 adapters are missing from the library, the sample cannot be sequenced because it will be unable to bind to the Illumina flow cell. In addition to the KAPA kit, qPCR kits from alternative vendors are also available that function according to the same principle. Oftentimes, researchers skip the qPCR check due to cost and time factors. In these instances, the Bioanalyzer data is used to check on the fragment size, and Qubit is used to quantify the library instead. This is the most commonly used approach, and Qubit is accurate for this purpose. However, please note that Qubit will not be able to provide information on whether the P5 and/or P7 adapters might be missing, while the Bioanalyzer traces might be too ambiguous for detection of missing sequence. Without quantification via qPCR, there will be no opportunity to notice that something might be wrong with the library until after sequencing.
25. *Estimated Number of Cells*. This number should be close to the targeted cell recovery number. If this number is far removed from the target, it could indicate an issue with cell handling (e.g., inaccurate cell counting), a problem that occurred during the run on the Chromium instrument, or that the cell type was significantly more fragile to work with than anticipated.

Fraction Reads in Cells This metric indicates the percentage of reads that are associated with a valid cell barcode and are confidently mapped to the transcriptome. This metric should be as high as possible, but ideally above 70%. A high percentage here means that the data are clean from background noise and that the sample contained healthy and viable cells. If the cell handling during the sample preparation upstream of the Single Cell Gene Expression assay had been too harsh, the cells could have died or ruptured, causing the release of their mRNA contents into the microcentrifuge tube. This ambient RNA will not be called as a cell by the Cell Ranger pipeline, therefore increasing the background noise and contaminating the data from partitions containing individual cells.

Barcode Rank Plot This graph plots all detected barcodes versus the total number of UMIs that have been counted for each one of those respective barcodes, ranking the barcodes from the ones with the highest UMI count to the ones with the lowest (Fig. 7). Interpretation of this plot is probably the most difficult one as an algorithm will usually have a hard time interpreting the shape of a curve, while the human eye can easily see patterns that the algorithm cannot calculate properly. The Barcode Rank Plot should

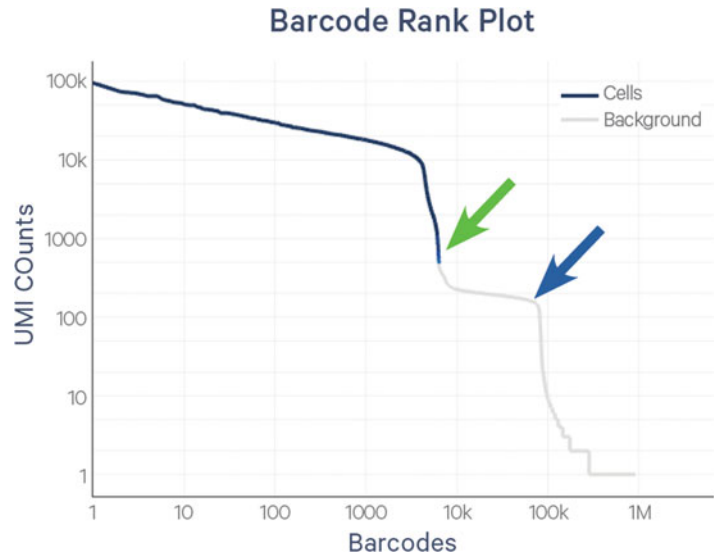

Fig. 7 Barcode Rank Plot in heterogeneous cell populations. Graph represents all detected barcode (x-axis) versus UMI counts (y-axis). Green arrow indicates the first typical sharp drop after the UMI-rich plateau (blue line), and blue arrow indicates a second drop in the form of a cliff (typical sign of a smaller cell population) in the background noise (gray line)

ideally have a plateau where the barcodes with the highest UMIs are located as this will contain the main bulk of the cells of the sample. From that UMI-rich plateau, we expect to find a sharp drop in the form of a cliff (green arrow, Fig. 7) into the background noise, i.e., the barcodes that contain very little or barely any UMIs. This would indicate the best separation between the actual single cell data and the noise. There will always be background noise in the data, but this should be kept to a minimum in order to avoid the ambient RNA contaminating the data from partitions containing real cells. When working with heterogeneous cell populations, there could be two cliffs before the plot drops off into the background noise. This is indicative of a smaller cell population that is presented within that lower second cliff. If the plot does not have a clearly defined plateau and cliff structure, this could be indicative of a wetting failure or a cell lysis event that caused the sample to lose its single cell characteristics.

Mean Reads Per Cell This is the sequencing depth of the sample, defined by the user when setting up the sequencing run. There is a minimum recommended number of reads per cell; however, this is strictly dependent on the cell type being processed. Large cells might require much higher sequencing depth than the minimum recommended amount.

Median Genes Per Cell and Median UMI Counts Per Cell These two values indicate data yield. Low numbers are related to the sequencing read depth in situations where the sequencing was too shallow for a given sample. However, if sequencing depth was not intentionally set this way, low numbers could be associated with lower cell viability, but they could also be cell type–dependent, e.g., when the cell is very small and/or not expressing that many transcripts. The numbers for these metrics should ideally be similar to previously processed samples of the same cell type.

Q30 Bases This is a summary of the Q-score ≥30 coming from the Illumina sequencer. These metrics should be as high as possible to ensure clean sequencing data; however, they are sequencing platform–dependent [22]. A drop in these metrics could indicate a potential problem with the sequencing run itself.

Sequencing Saturation This is the percentage of known reads, i.e., it is an indication of how deeply the sequencing has gone into the complexity of the library. This percentage depends on the cell type being processed as well as the targeted sequencing depth. Large cells are a lot more complex than small cells and therefore require more sequencing to reach the same level of saturation. The appropriate saturation will vary based on experimental needs. For some situations, a saturation level of 30% is sufficient for addressing the research objectives. On the other hand, sequencing very deep into the library—up to 90% saturation—may be preferred, in order to capture as much data from the cells as possible, allowing the identification of small differences between cell subtypes.

Reads Mapped Confidently to Transcriptome This is the percentage of reads that were able to be mapped to a unique gene in the reference transcriptome. These are the only reads which are used by Cell Ranger for UMI counting and therefore downstream secondary data analysis and gene expression calculations. How high this percentage needs to be, depends on the sample type, the species, and the quality of the reference file. Shortening the read length of Read 2 beyond the recommended length will negatively affect this metric.

Cell Cluster Interpretation Cell Clustering plots are the output from various clustering algorithms. The more similar the gene expression profile of the cells, the more likely they are going to be clustered together. Looking at the Cell Clustering plots requires a bit of background knowledge of the sample that was being processed, including the following: Was this a homogeneous or heterogeneous sample? What is the expectation for finding many cell types that are very different from each other, thereby forming many different clusters? If the Cell Cluster plots show one large, undefined mass of a cluster with a very homogeneous distribution of the

cells and/or UMI values, this could indicate a problem with the sample, such as a Wetting Failure or cell lysis prior to partitioning.

Gene Expression Table Interpretation The Gene Expression Table is the output of a differential gene expression analysis that is performed automatically by Cell Ranger for all clusters that have been detected in the previous step. The same types of questions from the Cell Cluster interpretation apply here: Was the sample type a homogeneous or heterogeneous mixture of various cell types? What is the expectation for finding large or small differences between the cell clusters?

Furthermore, identifying mitochondrial or apoptotic markers in this table is a sign that the cells were not in their best shape and had suffered in terms of viability.

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