10x Genomics®

Sample Preparation Demonstrated Protocols

Isolation of Nuclei for Single Cell RNA Sequencing

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CG000124 Rev B

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Single Nuclei Suspensions

These Demonstrated Protocols describe best practices and general protocols for cell lysis, washing, debris removal, counting, and concentrating nuclei from both single cell suspensions and neural tissue in preparation for use in 10x Genomics® Single Cell Protocols. Minimizing the presence of nuclear aggregates, dead cells, cellular debris, cytoplasmic nucleic acids, and potential inhibitors of reverse transcription is critical to obtaining high quality data.

The Protocols described here are expected to be compatible with many, but not all, cell or tissue types. Additional optimization may be required for the preparation of cell or tissue types that are particularly sensitive to suspension composition or handling techniques. Preparation of single cells or isolation of nuclei direct from solid tissues or cryopreserved samples may also require additional optimization during dissociation and/or cell handling not covered here. For additional information on preparation of specific sample types, consult 10x Genomics Demonstrated Protocols available on the 10x Genomics Support site support.10xgenomics.com.

Recommended Reagents & Equipment

Supplier	Description	Part Number (US)
-	Refrigerated Benchtop Centrifuge for 15 ml and 50 ml tubes	-
	Refrigerated Microcentrifuge for 2 ml LoBind tubes	-
Rainin	Tips LTS 200UL Filter RT-L200FLR	17007961
	Tips LTS W-O 200UL Filter RT-L200WFLR	17014294
	Tips LTS 1ML Filter RT-L1000FLR	17007954
	Tips LTS W-O 1ML Fltr RT-L1000WFLR	17014297
	Pipet-Lite LTS Pipette L-200XLS+	17014391
	Pipet-Lite LTS Pipette L-1000XLS+	17014382
Corning Cellgro	Phosphate-Buffered Saline (PBS) 1X without Calcium & Magnesium	21-040-CV
Sigma-Aldrich	Phosphate-Buffered Saline (PBS) with 10% Bovine Albumin *(alternative to Thermo Fisher product)*	SRE0036
	Trizma® Hydrochloride Solution, pH 7.4	T2194
	Sodium Chloride Solution, 5M	59222C
	Magnesium Chloride Solution, 1M	M1028
	Nonidet™ P40 Substitute	74385
	Protector RNase Inhibitor	3335399001
	Nuclei PURE 2M Sucrose Cushion Solution *(component of Nuclei PURE Prep Isolation Kit)*	NUC201-1KT
	Nuclei PURE Sucrose Cushion Solution *(component of Nuclei PURE Prep Isolation Kit)*	
	Live/Dead Cell Double Staining Kit	04511-1KT-F
Miltenyi Biotec	MACS® SmartStrainers, 30 µm	130-098-458
	Myelin Removal Beads II, Human, Mouse, Rat	130-096-733
	LS Columns	130-042-401
	MACS MultiStand	130-042-303
	QuadroMACS™ Separator *(for up to four simultaneous separations)*	130-090-976
	MidiMACS™ Separator *(for a single separation)*	130-042-302
Bel-Art	Flowmi™ Cell Strainer, 40 µm	H13680-0040
Thermo Fisher Sci	UltraPure™ Bovine Serum Albumin (BSA, 50 mg/ml)	AM2616
	Trypan Blue Stain (0.4%) for use with the Countess® Automated Cell Counter	T10282
	Countess® II FL Automated Cell Counter	AMAQAF1000
	Countess® II FL Automated Cell Counting Chamber Slides	C10228
Eppendorf	DNA LoBind Tubes 2.0 ml*	022431048
	DNA LoBind Tube 5.0 mL, PCR Clean	0030108310
Integra	PIPETBOY acu 2	155018
INCYTO	C-Chip™ Disposable Hemacytometer	DHCN012

Table continued on the next page

Supplier	Description	Part Number (US)
VWR	10 ml Serological Pipette	89130-898
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 50 ml	82018-050
	Sterile Polypropylene Centrifuge Tubes with Flat Caps, 15 ml	21008-103
	10 ml Serological Pipette	89130-898

*No substitutions are allowed. Items have been validated by 10x Genomics® and are required for Single Cell workflow, training and system operations.

Tips

Best Practices for Handling Biological Samples

Best practices for handling cell line/tissue samples include using sterile techniques, nuclease-free reagents and consumables, minimizing pipetting steps, and using wide-bore pipette tips when possible to minimize cell damage. Transfer pipettes may be used to remove supernatant after centrifugation to minimize disturbance of the cell/nuclei pellet.

Cells carry potentially hazardous pathogens. Follow material supplier recommendations and local laboratory procedures and regulations for the safe handling, storage and disposal of biological materials.

Factors Influencing Nuclei Recovery

These Demonstrated Protocols require suspensions of viable, single nuclei as input. To recover the expected number of nuclei, it is critical to maximize input cell viability, minimize cell and nuclei handling time, accurately count nuclei, and pipette the correct volume into the Single Cell Master Mix when executing 10x Genomics® Single Cell Protocols. Consult Technical Note *Guidelines for Accurate Target Cell Counts Using 10x Genomics® Single Cell Solutions* (Document CG000091) for more details.

Input Cell Suspension Quality

Input cell suspensions used in these Protocols should contain more than 90% viable cells. The presence of a high fraction of non-viable or dying cells may decrease recovery. The presence of ambient RNA and cellular debris may also impact application performance and negatively impact quality metrics reported by Cell Ranger™, including the "Fraction Reads in Cells". To increase sample viability and reduce the fraction of ambient RNA in the suspension, consult Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093).

Depending on cell type, viability may significantly decrease when cells are kept in suspension for a prolonged period before use. Furthermore, some cells types, including peripheral blood mononuclear cells (PBMCs), may form aggregates when kept in PBS for an extended period, thereby decreasing the effective concentration of single cells in the suspension. Therefore, additional washing and straining steps may be necessary to remove excess amount of ambient RNA and cellular aggregates, respectively.

As with most primary samples, the tissue types discussed in this Protocol are particularly fragile and extra care should be taken to preserve sample integrity. In addition, primary tissues may require additional handling steps (*e.g.* flow cytometry, filtration) to maximize input sample quality and specificity.

Lysis

Isolation of nuclei from cells requires disrupting the structural integrity of the mammalian cellular membrane. Detergent-based lysis solubilizes the hydrophobic membrane proteins which releases the cell's cytoplasmic contents (*i.e.* organelles, cytoplasmic nucleic acids, ambient RNA, proteases) without impacting the integrity of the subcellular components. A combination of low speed centrifugation and repeated washing steps then separates the nuclei from other organelles, nuclear aggregates, dead cells, cellular debris, cytoplasmic nucleic acids, and potential inhibitors of reverse transcription.

Centrifugation speed/time, number of wash steps, and methods of debris removal (*e.g.* filtration, myelin removal, or flow cytometry) may require optimization for a specific sample type. For example, solid tissues and cryopreserved samples may require preparation prior to lysis and therefore, the lysis time will likely be different for each sample type.

Lysis Time & Temperature

Lysis efficacy should be assessed via microscopy after 3 – 5 min for single cell suspensions or ~15 min for neural tissue. When working with new cell/tissue types, it is recommended to optimize lysis time. The presence of a high fraction of viable cells will impact application performance and therefore, it is important to maximize the extent of lysis efficiency. Cell lysis should be carried out on ice and using chilled reagents.

Washing & Resuspension

Purifying isolated nuclei via washing minimizes excess cellular debris, cytoplasmic nucleic acids, and potential inhibitors of reverse transcription.

When washing and resuspending nuclei, always use sufficient volumes to maintain concentrations of less than 5000 nuclei/µl (*i.e.* 5 million nuclei resuspended in 1 ml Nuclei Washing and Resuspension Buffer). Maintaining nuclei at higher concentrations may cause aggregation and clumping.

The recommended Nuclei Washing and Resuspension Buffer contains BSA, to minimize nuclei losses and aggregation, and RNase Inhibitor to inhibit RNases during nuclei isolation and purification.

The presence of BSA and RNase Inhibitor in the Nuclei Washing and Resuspension Buffer results in a translucent/opaque rather than clear aqueous phase after breaking the GEMs with Recovery Agent. This is normal and will not impact downstream operations. See Section 5 – Troubleshooting for more details.

Regular-Bore versus Wide-Bore Pipette Tips

Using roughly treated or damaged cells as input to these Protocols may compromise system performance. It is particularly important to pipette cell suspensions gently when using a regular-bore pipette tip as the smaller diameter is more likely to shear and damage cells. The speed at which the cells pass through the opening of the pipette tip is also important – the faster cells pass through, the more likely they will shear and prematurely lyse.

To avoid cellular shearing and premature lysis, the use of wide-bore pipette tips is recommended for most pipetting steps. However, generating single nuclei suspensions from pellets or clumps is best achieved using a regular-bore pipette tip to break up aggregates. To minimize physical damage to nuclei from shearing forces, it is critical to pipette gently and slowly during resuspension steps.

Centrifugation Conditions

Depending on the total nuclei concentration, centrifugation will result in a visible nuclei pellet with minimal nuclei remaining in the supernatant. Avoid excessive centrifugation speeds and times as this may result in nuclei damage. Moreover, a tightly packed nuclei pellet may require additional pipetting to achieve complete resuspension, which may decrease nuclei integrity.

Recommended centrifugation conditions for samples used in these Protocols is 500 rcf for 5 min at 4°C. This will maintain the integrity of the nuclear envelope and maximize recovery. When working with a new sample type, save and count nuclei in the supernatant to ensure that losses are minimal. Optimize centrifugation conditions to minimize nuclei loss while preserving sample integrity (*e.g.* 600 rcf and/or 8 min at 4°C).

Aggregate & Debris Removal – Straining

Filtering lysed tissues and nuclei suspensions with an appropriate cell strainer helps to remove cellular debris and nuclei aggregates. Strainers with appropriate pore sizes should be used to allow nuclei to pass through the filter while cellular debris and nuclei aggregates are retained.

Depending on the degree of aggregation and the strainer type, the number of nuclei and the amount of wash solution retained in the strainer can vary. The MACS® SmartStrainer is recommended as it generally causes minimal changes to the nuclei concentration. However, a volume loss of 100 µl or more can occur. For low nuclei suspension volumes, the Flowmi™ Tip Strainer is recommended to minimize volume losses. Nuclei concentrations may decrease by up to 40%, depending on suspension volume and strainer type. It is therefore important to measure the concentration of the nuclei suspension before and after straining.

Aggregate & Debris Removal – Myelin Removal

Myelin, the membrane formed by glial cells that surrounds and insulates axons in the peripheral and central nervous system, begins formation during embryonic development in humans and around birth in mice and rapidly accumulates as the nervous system matures.

When lysing neural tissue, large quantities of myelin debris are produced. Depending on the age of the tissue, reduction of myelin debris may improve the cleanliness of final nuclei preparation.

The recommended method for myelin removal is via Myelin Removal Beads II (Human, Mouse, Rat), in combination with chilled reagents.

Aggregate & Debris Removal – Density Gradient Centrifugation

Density gradient (sucrose) centrifugation is commonly used to isolate sub-cellular components or remove contaminants and debris.

Nuclei isolated from different cell types or tissues may require optimization of the sucrose concentrations or centrifugation speeds and times used in this Protocol to maximize recovery and purity.

Nuclei Counting

Visualization of nuclei suspensions is critical for accurate determination of nuclei concentration and viability, suspension quality, and nuclei sizes prior to use in 10x Genomics® Single Cell Protocols.

The Countess® II FL Automated Cell Counter is recommended for determining nuclei concentrations for most applications. Sample types with very small nuclei or high levels of aggregation may require alternative counting methods.

When characterizing a sample type for the first time, it is recommended to perform two different counting and viability assays. The first recommended method is to stain nuclei with trypan blue and count the nuclei concentration and viability using the Countess II FL Automated Cell Counter. The second recommended method is to stain nuclei with fluorescent dyes and measure cell viability using a tissue culture microscope and automated cell counting software. Manual counting using a hemocytometer may be used as an additional method.

Nuclei suspensions should also be checked visually for debris or nuclei aggregates as these can clog microfluidic channels. If observed, additional pipetting or filtering may be required to obtain optimal performance.

Concentration of Nuclei for Optimal Performance

The total number of suspended nuclei used as input to 10x Genomics® Single Cell Protocols is determined by the nuclei recovery target. Consult the applicable 10x Genomics Single Cell Protocol to determine these relationships. To maximize the likelihood of achieving the desired recovery target, the optimal input nuclei concentration is 700 – 1200 nuclei/µl.

If possible, bring the input nuclei suspension to a concentration that is optimal for the dynamic range of counting technique used (manual or automated), allows for 3 – 4 reproducible counts (where the standard deviation of these counts is <25%), and requires pipetting 2.5 – 15 µl of the nuclei suspension into the Single Cell Master Mix. Pipetting nuclei suspension volumes <2.5 µl increases variance due to pipetting inaccuracy, while volumes >15 µl increases the risk of introducing unwanted debris or inhibitors.

It is critical to estimate the number of input nuclei from the final single nuclei suspension because nuclei are inevitably lost during washing and resuspension steps.

Flow Cytometry of Single Nuclei

If a sample concentration and volume allows, flow cytometry may further improve the purity of nuclei suspensions used as input for the 10x Genomics Single Cell Protocols. It is critical to visually inspect the sorted nuclei under a microscope and re-count the nuclei suspension using a cell counter or hemocytometer prior to pipetting into the Single Cell Master Mix. Nuclei counts that are based on the flow cytometer are inaccurate.

Nuclei Control Sample

To measure success and assess the reproducibility of these Protocols, it is recommended to run a quality control sample in parallel with each experimental sample. Recommended quality control samples include cultured cell lines (*e.g.* human HEK293T cells) that are of high quality (>90% viable) and yield sufficient number of nuclei (*i.e.* >1 million nuclei).

Isolation of Nuclei from Single Cell Suspensions

Protocol

1. Isolation of Nuclei from Single Cell Suspensions

1.1. Overview

This Demonstrated Protocol outlines how to isolate, wash, and count single nuclei from prepared single cell suspensions in preparation for use in 10x Genomics® Single Cell Protocols. Modifications to this Protocol may be required when working with new sample types for the first time (*e.g.* lysis time, centrifugation speed/time, and filtration).

This Protocol assumes that the input single cell suspensions are prepared, washed and counted as described in the applicable 10x Genomics Demonstrated Protocol:

- *Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing* (Document CG00054)
- *Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing* (Document CG00014)
- *Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing* (Document CG00039)
- *Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing* (Document CG00055)

1.2. Preparation – Buffers

a) Prepare chilled (4°C) Lysis Buffer: 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ and 0.005% Nonidet P40 in Nuclease-Free Water.

NOTE

Lysis Buffer may be stored for up to a week at 4°C.

b) Prepare chilled (4°C) Nuclei Wash and Resuspension Buffer: 1X PBS with 1.0% BSA and 0.2U/µl RNase Inhibitor.

1.3. Specific Cells & Tissue Sourcing

NOTE

This Protocol was demonstrated with the cells outlined below, including cells dissociated from fresh E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone tissue from BrainBits (www.brainbitsllc.com). Materials were stored according to manufaturer's recommendations prior to starting the Protocol. Fresh embryonic mouse brain tissue was shipped on cold packs and used immediately upon receipt.

Cell Type	Species	Supplier	Part Number
Jurkat, Clone E6-1	Human	ATCC	ATCC® TIB-152™
293T/17 [HEK 293T/17]	Human	ATCC	ATCC® CRL-11268™
NIH/3T3	Mouse	ATCC	ATCC® CRL-1658™
Normal Peripheral Blood MNC	Human	All Cells	PB002
E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone	Mouse	Brain Bits	C57EHCV

1.4. Nuclei Isolation

NOTE

The optimal input concentration of single cells for this Protocol is 2.5 x 10^6 total cells. This Protocol takes ~35 min to complete.

a) Centrifuge the cells at **300 rcf** for **5 min**.

b) Remove the supernatant without disrupting the cell pellet.

c) Using a **wide-bore** pipette tip, add **1 ml** Lysis Buffer and gently pipette mix 5 times or until cells are completely suspended.

d) Lyse the cells on **ice** for **5 min**.

e) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

f) Remove the supernatant without disrupting the nuclei pellet.

g) Using a **regular-bore** pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 5 times.

CRITICAL!

When working with new cell/tissue types, it is recommended to optimize lysis time. At this point, lysis efficacy should be assessed via staining the nuclei with trypan blue and viability should be assessed using the Countess® II FL Automated Cell Counter/microscopy. If a high fraction of viable cells is still present, repeat steps d – g, incrementally increasing the lysis time and monitoring efficacy via microscopy.

Repeat

h) Repeat steps e – g.

i) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

j) Remove the supernatant without disrupting the nuclei pellet.

k) Using a **regular-bore** pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer or an appropriate volume to the pelleted nuclei to achieve the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml). Gently pipette mix 8 – 10 times or until nuclei are completely suspended.

l) Use a cell strainer to remove cell debris and large clumps. For low volume, a **40 µm** Flowmi™ Tip Strainer is recommended to minimize loss of sample volume.

NOTE

To increase the efficiency of debris removal, the nuclei suspension may be filtered twice.

m) Determine the nuclei concentration using a Countess II FL Automated Cell Counter or hemocytometer.

n) If the nuclei concentration is **<500 nuclei/µl** (5 x 10^5 nuclei/ml), adjust the volume accordingly.

o) Once the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml) is obtained, place the nuclei on ice.

p) Proceed immediately with the 10x Genomics® Single Cell Protocol and minimize the time between nuclei preparation and chip loading.

Isolation of Nuclei from Embryonic Mouse Brain Tissue

Protocol

2. Isolation of Nuclei from Embryonic Mouse Brain Tissue

2.1. Overview

This Demonstrated Protocol outlines how to isolate, wash, and count single nuclei from embryonic mouse brain tissue sections in preparation for use in 10x Genomics® Single Cell Protocols. Modifications to this Protocol may be required when working with new sample types for the first time (*e.g.* lysis time, centrifugation speed/time, debris removal, and filtration steps).

2.2. Preparation – Buffers

a) Prepare chilled (4°C) Lysis Buffer: 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ and 0.005% Nonidet P40 in Nuclease-Free Water.

b) Prepare chilled (4°C) Nuclei Wash and Resuspension Buffer: 1X PBS with 1.0% BSA and 0.2U/µl RNase Inhibitor.

c) Prepare chilled (4°C) LS Column Calibration Buffer: 1X PBS with 0.5% BSA.

2.3. Specific Tissue Sourcing & Materials

NOTE

This Protocol was demonstrated with fresh E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone tissue from BrainBits (www.brainbitsllc.com). Materials were stored according to manufaturer's recommendations prior to starting the Protocol. Fresh embryonic mouse brain tissue was shipped on cold packs and used immediately upon receipt.

Tissue / Material	Description	BrainBits SKU
Neuronal Tissue	E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone	C57EHCV
Fire Polished Silanized Pasteur Pipette	-	FPP

2.4. Tissue Lysis & Washing of Nuclei

NOTE

This step of the Protocol takes ~40 min to complete.

a) Using a 1000 µl **wide-bore** pipette tip, gently transfer the tissue along with the Hibernate E®/B27®/GlutaMAX™ (HEB) medium to a new 15 ml conical tube and wait until the tissue is settled at the bottom of the tube.

b) Transfer the HEB medium from the tissue to a new 15 ml conical tube, leaving only enough medium to cover the tissue. Keep the HEB medium **on ice** for step d.

c) Add **2 ml** chilled Lysis Buffer to the tissue and lyse the tissue on **ice** for **15 min**. Gently swirl to mix, repeat 2 – 3 times during the incubation.

d) Add the HEB medium saved from step b back to the lysed tissue.

e) Aspirate the tissue and the HEB medium into a fire polished silanized Pasteur pipette and immediately dispense the contents back into the tube. Triturate with 5 – 7 passes of the tissue through the pipette.

f) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

g) Remove the supernatant without disrupting the nuclei pellet.

h) Using a **regular-bore** pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 8 – 10 times.

CRITICAL!

*When working with new cell/tissue types, it is recommended to optimize lysis time. At this point, lysis efficacy should be assessed via staining the nuclei with trypan blue and viability should be assessed using the Countess® II FL Automated Cell Counter/microscopy. If a high fraction of viable cells is still present, centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**, add **2 ml** chilled Lysis Buffer and incrementally increase the lysis time, monitoring efficacy via microscopy. When optimal lysis has occurred, repeat steps f – h.*

i) Use a **40 µm** Flowmi™ Cell Strainer to remove cell debris and large clumps. Transfer to a 2 ml centrifuge tube.

j) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

k) Remove the supernatant without disrupting the nuclei pellet.

l) Using a **regular-bore** pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 8 – 10 times.

Repeat

m) Repeat steps i – k.

n) Using a **regular-bore** pipette tip, add **180 µl** Nuclei Wash and Resuspension Buffer and gently pipette mix 8 – 10 times.

NOTE

The volume of buffer used for resuspension will depend on the age and mass of the input tissue and the volume of Myelin Removal Beads II required in step 2.5. Both should be adjusted according to the manufacturer's instructions.

o) Proceed directly to Myelin Removal.

2.5. Myelin Removal

This step of the Protocol takes ~45 min to complete.

NOTE

This Protocol was demonstrated using sample sizes compatible with Myelin Removal Beads II and a single LS Column. The volumes of buffer, Myelin Removal Beads II, and number of LS columns depend on the age and mass of the tissue and should be adjusted according to the manufacturer's instructions.

a) Add **20 µl** Myelin Removal Beads II to the resuspended nuclei from step 2.4n. Mix thoroughly with a **wide-bore** pipette tip. Do not vortex.

b) Incubate for **15 min** at **4°C**.

c) Meanwhile, prepare an LS column with **3 ml** LS Column Calibration Buffer.

d) After incubation is complete, dilute the nuclei suspension (containing Myelin Removal Beads II) with **5 ml** Nuclei Wash and Resuspension Buffer (using a 10 ml serological pipette) and gently pipette mix 5 times.

e) Centrifuge the nuclei at **500 rcf** for **10 min** at **4°C**.

f) Remove the supernatant without disrupting the nuclei pellet.

g) Resuspend the pelleted nuclei in **1 ml** Nuclei Wash and Resuspension Buffer.

h) Apply the resuspended nuclei to the LS column.

i) Wash the column **twice** with **1 ml** Nuclei Wash and Resuspension Buffer.

j) Collect the effluent in one 5 ml Eppendorf tube.

k) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

l) Remove the supernatant without disrupting the nuclei pellet.

m) Using a **regular-bore** pipette tip, add **500 µl** Nuclei Wash and Resuspension Buffer or an appropriate volume to the pelleted nuclei to achieve the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml). Gently pipette mix 8 – 10 times or until nuclei are completely suspended.

n) Use a cell strainer to remove cell debris and large clumps. For low volume, a **40 µm** Flowmi™ Tip Strainer is recommended to minimize loss of sample volume.

o) Determine the nuclei concentration using a Countess® II FL Automated Cell Counter or hemocytometer.

p) If the nuclei concentration is **<500 nuclei/µl** (5 x 10^5 nuclei/ml), adjust the volume accordingly.

q) Once the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml) is obtained, place the nuclei on ice.

r) Proceed immediately with the 10x Genomics® Single Cell Protocol and minimize the time between nuclei preparation and chip loading.

Isolation of Nuclei from Adult Mouse Brain Tissue

Protocol

3. Isolation of Nuclei from Adult Mouse Brain Tissue

3.1. Overview

This Demonstrated Protocol outlines how to isolate, wash, and count single nuclei from adult mouse brain tissue sections in preparation for use in 10x Genomics® Single Cell Protocols. Modifications to this Protocol may be required when working with new sample types for the first time (*e.g.* lysis time, centrifugation speed/time, debris removal, and filtration steps).

3.2. Preparation – Buffers

a) Prepare chilled (4°C) Lysis Buffer: 10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$ and 0.005% Nonidet P40 in Nuclease-Free Water.

b) Prepare chilled (4°C) Nuclei Wash and Resuspension Buffer: 1X PBS with 1.0% BSA and 0.2U/µl RNase Inhibitor.

c) Prepare chilled (4°C) LS Column Calibration Buffer: 1X PBS with 0.5% BSA.

d) Prepare Sucrose Cushion Buffer I: 2.7 ml Nuclei PURE 2M Sucrose Cushion Solution with 300 µl Nuclei PURE Sucrose Cushion Solution.

3.3. Specific Tissue Sourcing & Materials

NOTE

The Protocol was demonstrated with fresh Adult Mouse Combined Cortex, Hippocampus and Ventricular Zone tissue from BrainBits (www.brainbitsllc.com). Materials were stored according to manufaturer's recommendations prior to starting the Protocol. Fresh adult mouse brain tissue was shipped on cold packs and used immediately upon receipt.

Tissue / Material	Description	BrainBits SKU
Neuronal Tissue	Adult Mouse Combined Cortex, Hippocampus and Ventricular Zone	C57AHCV
Fire Polished Silanized Pasteur Pipette	-	FPP

3.4. Tissue Lysis & Washing of Nuclei

NOTE

This step of the Protocol takes ~45 min to complete.

a) Using a 10 ml serological pipette, gently transfer the tissue along with the Hibernate A®/B27®/GlutaMAX™ (HEB) medium to a new 50 ml conical tube and wait until the tissue is settled at the bottom of the tube. Only pipette as much HEB media needed to isolate the tissue.

b) Transfer the HEB medium from the tissue to a new 15 ml conical tube, leaving only enough medium to cover the tissue. Keep the HEB medium on **ice** for step d.

c) Add **5 ml** chilled Lysis Buffer to the tissue and incubate on **ice** for **15 min**. Gently swirl to mix, and repeat 2 – 3 times during the incubation.

d) Add **5 ml** HEB medium saved from step b back to the lysed tissue.

e) Aspirate the tissue and the HEB medium into a fire polished silanized Pasteur pipette and immediately dispense the contents back into the tube. Triturate with 10 – 15 passes of the tissue through the pipette.

f) Use a **30 µm** MACS® SmartStrainer to remove cell debris and large clumps.

g) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

h) Remove the supernatant without disrupting the nuclei pellet.

i) Using a 10 ml serological pipette add **10 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 8 – 10 times.

CRITICAL!

*When working with new cell/tissue types, it is recommended to optimize lysis time. At this point, lysis efficacy should be assessed via staining the nuclei with trypan blue and viability should be assessed using the Countess® II FL Automated Cell Counter/microscopy. If a high fraction of viable cells is still present, centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**, add **10 ml** chilled Lysis Buffer and incrementally increase the lysis time, monitoring efficacy via microscopy. When optimal lysis has occurred, repeat steps f – i.*

j) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

k) Use a **30 µm** MACS® SmartStrainer to remove cell debris and large clumps.

l) Using a 10 ml serological pipette, add **5 ml** Nuclei Wash and Resuspension Buffer and gently pipette mix 8 – 10 times.

m) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

n) Remove the supernatant without disrupting the nuclei pellet.

o) Using a **regular-bore** pipette tip, add **1080 ml** Nuclei Wash and Resuspension Buffer to the pelleted nuclei and gently pipette mix 8 – 10 times or until nuclei are completely suspended.

NOTE

The volume of buffer used for resuspension will depend on the age and mass of the input tissue and the volume of Myelin Removal Beads II required in step 3.5. Both should be adjusted according to the manufacturer's instructions.

p) Proceed directly to Myelin Removal.

3.5. Myelin Removal

This step of the Protocol takes ~45 min to complete.

NOTE

This Protocol was demonstrated using sample sizes compatible with Myelin Removal Beads II and two LS Columns. The volumes of buffer, Myelin Removal Beads II, and number of LS columns depend on the age and mass of the tissue and should be adjusted according to the manufacturer's instructions.

a) Add **120 µl** Myelin Removal Beads II to the resuspended nuclei from step 3.4o. Mix thoroughly with a **wide-bore** pipette tip. Do not vortex.

b) Incubate for **15 min** at **4°C**.

c) Meanwhile, prepare two LS columns, each with **3 ml** LS Column Calibration Buffer.

d) After incubation is complete, dilute the nuclei suspension (containing Myelin Removal Beads II) with **10 ml** Nuclei Wash and Resuspension Buffer (using a 10 ml serological pipette) and gently pipette mix 5 times.

e) Centrifuge the nuclei at **500 rcf** for **10 min** at **4°C**.

f) Remove the supernatant without disrupting the nuclei pellet.

g) Resuspend the pelleted nuclei in **2 ml** Nuclei Wash and Resuspension Buffer and proceed to magnetic separation.

h) Apply **1 ml** nuclei suspension to each LS column.

i) Wash each of the columns **twice** with **1 ml** Nuclei Wash and Resuspension Buffer.

j) Collect the effluent into two 5 ml Eppendorf tubes.

k) Centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**.

l) Remove the supernatant without disrupting the nuclei pellet.

m) Resuspend the contents of each Eppendorf tube in **500 µl** Nuclei Wash and Resuspension Buffer.

n) Determine the nuclei concentration using a Countess® II FL Automated Cell Counter or hemocytometer.

NOTE

*If the concentration in each tube is <1 x 10^7 total nuclei, combine the contents of each tube, centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**, remove the supernatant without disrupting the nuclei pellet and resuspend in **500 µl** Nuclei Wash and Resuspension Buffer.*

o) Proceed to Density Gradient Centrifugation.

3.6. Density Gradient Centrifugation

This step of the Protocol takes ~1 h to complete.

This step assumes that >1 x 10^7 total nuclei were obtained in each of the Eppendorf tubes in step 3.5n.

NOTE

*If <1 x 10^7 total nuclei were obtained, combine the contents of each tube, centrifuge the nuclei at **500 rcf** for **5 min** at **4°C**, remove the supernatant without disrupting the nuclei pellet, and resuspend in 500 µl Nuclei Wash and Resuspension Buffer. Then proceed by preparing the sucrose gradient in a single tube.*

a) Add **900 µl** Sucrose Cushion Buffer I to each Eppendorf tube containing **500 µl** of resuspended nuclei from step 3.5m. Pipette mix 10 times using a **regular-bore** pipette tip.

b) Prepare two sucrose gradients by adding **500 µl** Sucrose Cushion Buffer I to two 2 ml Eppendorf tubes.

c) Carefully layer each of the **1400 µl** nuclei suspensions from step a to the top of each tube containing Sucrose Cushion Buffer I. Do not mix.

d) Centrifuge the sucrose gradient containing the nuclei at **13000 x g** for **45 min** at **4°C**.

e) Carefully remove supernatant leaving **100 µl** in each tube. Using a **regular-bore** pipette tip, resuspend the nuclei pellets.

f) Using a **regular-bore** pipette tip, add **1 ml** Nuclei Wash and Resuspension Buffer or an appropriate volume to achieve the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml). Gently pipette mix 8 – 10 times or until nuclei are completely suspended.

g) Use a **40 µm** Flowmi™ Cell Strainer to remove cell debris.

h) Determine the nuclei concentration using a Countess® II FL Automated Cell Counter or hemocytometer.

i) If nuclei concentration is **<500 nuclei/µl** (5 x 10^5 nuclei/ml), adjust the volume accordingly.

j) Once the target nuclei concentration of **1000 nuclei/µl** (1 x 10^6 nuclei/ml) is obtained, place the nuclei on ice.

k) Proceed immediately with the 10x Genomics® Single Cell Protocol and minimize the time between nuclei preparation and chip loading.

Results

4. Results

4.1. Sample Preparation – Using a Countess® II FL Automated Cell Counter

NOTE

Refer to the manufacturer's instructions for details on the operation of the Countess® II FL Automated Cell Counter. The optimal range of cell concentration for this device is 100 to 4000 cells/µl.

a) Thoroughly vortex 0.4% trypan blue stain and centrifuge briefly.

b) Using a **wide-bore** pipette tip, gently and thoroughly mix the nuclei.

NOTE

It is critical that the nuclei suspension is homogeneous to minimize sampling error. Also ensure that nuclei are free of debris and fibers as these can interfere with nuclei counting.

c) Immediately aliquot **10 µl** nuclei and add **10 µl** 0.4% trypan blue stain. Gently pipette mix.

d) Transfer **10 µl** trypan blue stained nuclei to a chamber on Countess II Cell Counting Slide.

e) Insert the slide into the Countess II FL Cell Counter, and determine the nuclei concentration and viability.

NOTE

<5% of input cells should be viable. Optimization of focusing and light exposure are critical for accurate counting.

4.2. Sample Preparation – Using Fluorescent Viability Stains & Microscopy

NOTE

The Live/Dead Cell Double Staining Kit is used for simultaneous fluorescence staining of viable and dead cells. Refer to the manufacturer's instructions for details. Other kits or dyes may require fluoresence filters with alternative excitation and emission ranges.

Prepare fresh staining solution immediately before use.

a) Bring the tubes of Calcein AM and Propidium Iodide to **room temperature**.

b) Vortex both tubes and centrifuge briefly.

c) Add **10 µl** Calcein AM (Solution A) and **5 µl** Propidium Iodide (Solution B) to **5 ml** 1X PBS (calcium, magnesium free) and vortex for **5 sec**.

d) Immediately aliquot **25 µl** cells or nuclei (prepared at a concentration of **1000 cells or nuclei/µl** (1 x 10^6 nuclei/ml)) and add **25 µl** fluorescent staining solution. Pipette mix gently.

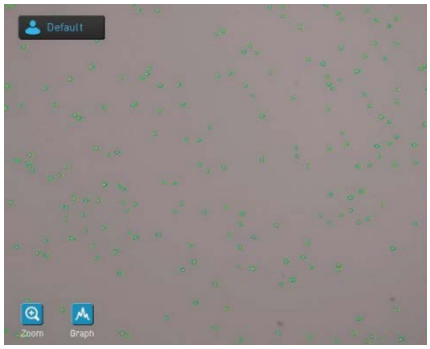
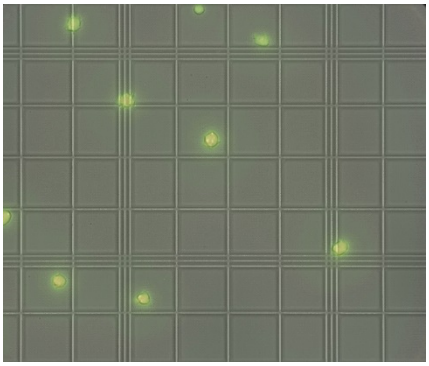
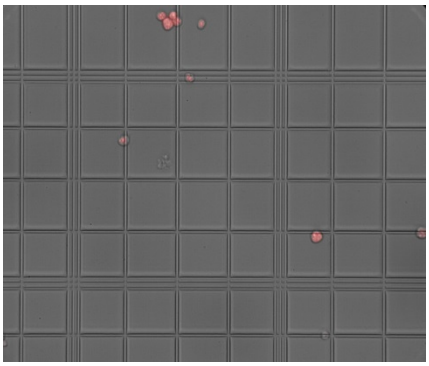
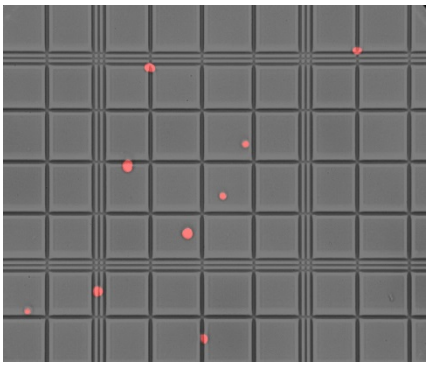
e) Incubate the mixture at **37°C** for **15 min**.

f) Load **10 µl** of the stained cells or nuclei onto a slide (e.g. C-Chip™ Disposable Hemacytometer).

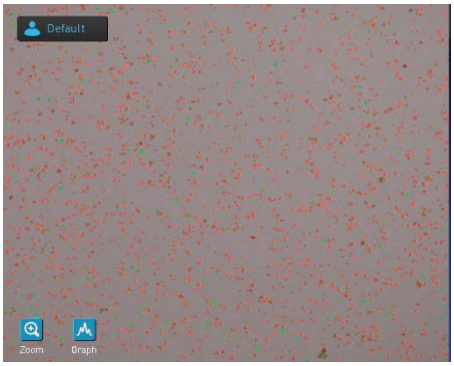
g) Image cells using a microscope with a 20X or 40X objective.

h) Detect fluorescence using a fluorescence microscope with λ490 nm excitation for simultaneous monitoring of viable and dead cells or nuclei. Use λ545 nm excitation to visualize non-viable/dead cells or nuclei only.

4.3. Sample Preparation – Isolation of Nuclei from Single Cell Suspensions

Jurkat Cells – Representative Images of Staining on a Countess® II FL Automated Cell Counter	Jurkat Cells – Representative Images of Staining on a Hemocytometer
 Pre lysis	 Pre lysis
 Post lysis	 Post lysis
 Post lysis, washing and filtration	 Post lysis, washing and filtration

4.4. Sample Preparation – Isolation of Nuclei from Adult Mouse Brain Tissue

Adult Mouse Combined Cortex, Hippocampus and Ventricular Zone – Representative Images of Staining on a Countess® II FL Automated Cell Counter
 Post Tissue Dissociation and Lysis
 Post Myelin Removal
 Post Myelin Removal and Density Gradient Centrifugation

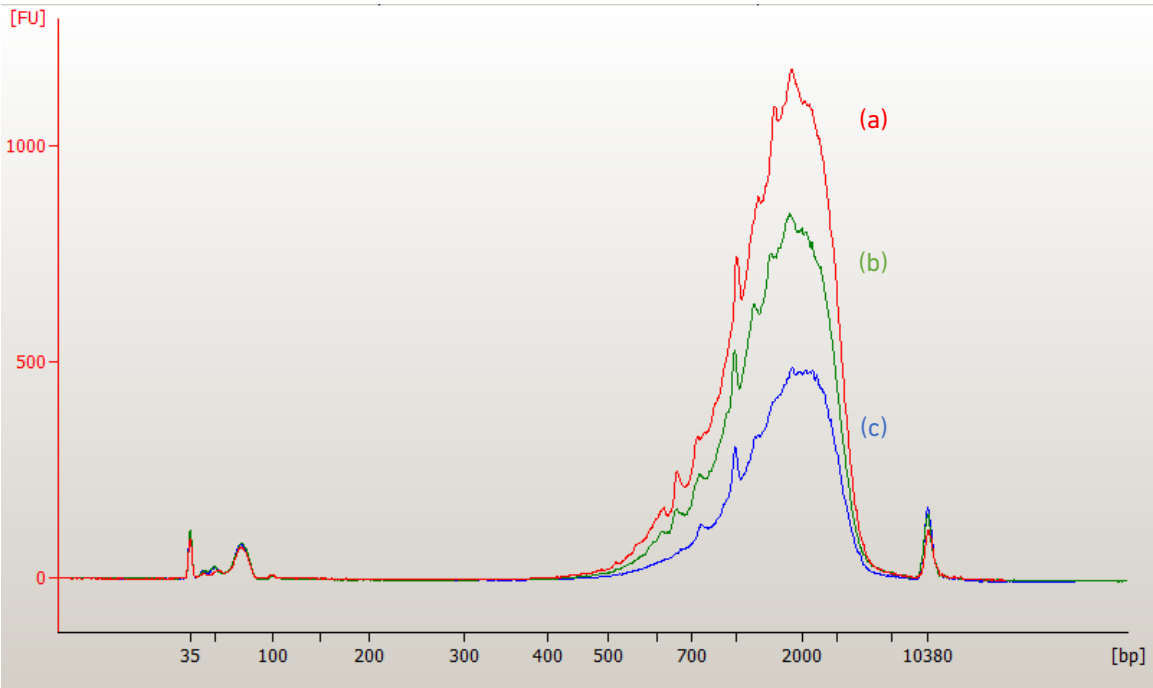
4.5. Partitioning and Library Preparation

A B C D E F G H

After transfer of the GEMs + Recovery Agent to a tube strip:

- Tube A: Cell sample suspended in 1x PBS + 0.04% BSA – the aqueous phase appears clear.
- Tubes B-H: Samples containing different input volumes of nuclei suspended 1x PBS + 1.0% BSA + 0.2U/µl – the aqueous phases may appear translucent/opaque. This is normal.

4.6. Post cDNA Amplification



1 µl of sample undiluted on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis. Traces should resemble the overall shape of the sample electropherograms above.

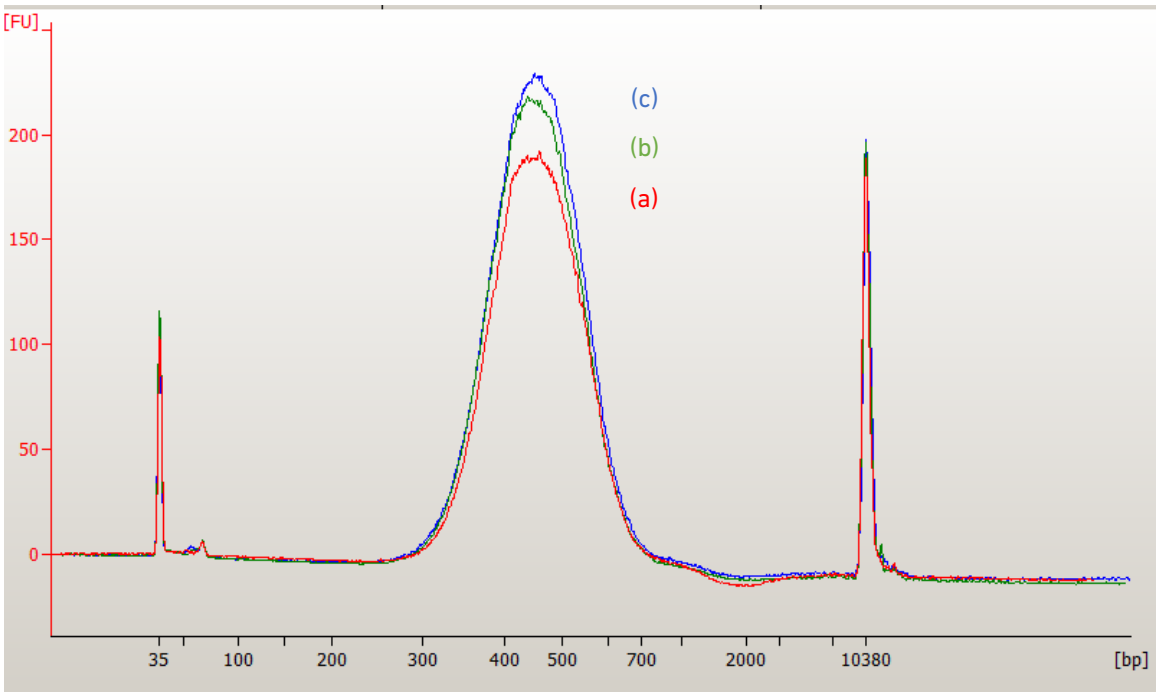
(a) 2000 nuclei HEK293T nuclei isolated according to the Isolation of Nuclei from Single Cell Suspensions Protocol (Section 1).

(b) 2000 E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone nuclei isolated according to the Isolation of Nuclei from Embryonic Mouse Brain Tissue Protocol (Section 2).

(c) 2000 E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone nuclei isolated from a dissociated single cell suspension, according to the Isolation of Nuclei from Single Cell Suspensions Protocol (Section 1).

Note: Each of the samples went through 14 cycles of cDNA Amplification.

4.7. Post Library Construction QC



1 µl of sample at a dilution of 1 part sample:9 parts Nuclease-Free Water on the Agilent Bioanalyzer High Sensitivity chip for qualitative analysis. Traces should resemble the overall shape of the sample electropherograms above.

(a) 2000 nuclei HEK293T nuclei isolated according to the Isolation of Nuclei from Single Cell Suspensions Protocol (Section 1).

(b) 2000 E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone nuclei isolated according to the Isolation of Nuclei from Embryonic Mouse Brain Tissue Protocol (Section 2).

(c) 2000 E18 Mouse Combined Cortex, Hippocampus and Ventricular Zone nuclei isolated from a dissociated single cell suspension, according to the Isolation of Nuclei from Single Cell Suspensions Protocol (Section 1).

Troubleshooting & References

5. Troubleshooting

5.1. Troubleshooting Sample Preparation

Problem	Possible Reason	Solution
High fraction of non-viable **cells** in input material prior to starting nuclei preparation	• Poor tissue quality and sub-optimal tissue dissociation protocol • Fragile sample type • Rough cell handling (fast pipetting, use of regular-bore pipette tips) • Prolonged cell preparation time (>30 min)	• Optimize cell/tissue dissociation protocol for improved sample quality • Reduce fraction of dead cells following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Gently handle cell suspensions by following best practices • Reduce cell processing time
	• High fraction of cellular debris in final resuspension	• Add 1 – 2 additional wash steps • Filter cell suspension with the appropriate strainer • Use flow cytometry to sort sample
High fraction of viable cells post lysis	• Incomplete cell lysis • Suboptimal lysis conditions	• Incrementally increase lysis time and monitor efficacy using microscopy
High fraction of visible debris post lysis	• Insufficient removal of debris	• Filter nuclei suspension with the appropriate strainer • Remove myelin and other impurities with myelin removal beads and/or density gradient centrifugation • Use flow cytometry to sort sample
<700 nuclei/µl after final resuspension	• Low input cell number prior to cell lysis • Overly dilute nuclei suspension	• Concentrate nuclei suspension to achieve target concentration of 700 – 1200 nuclei/µl
>1200 nuclei/µl after final resuspension	• Overly concentrated nuclei suspension	• Dilute nuclei suspension to achieve target concentration of 700 – 1200 nuclei/µl

5.2. Troubleshooting Partitioning & Library Preparation

Problem	Possible Reason	Solution
No clear aqueous solution post GEM breaking with Recovery Agent	• Elevated protein concentration in nuclei suspension media	• Proceed with the 10x Genomics® Single Cell Protocol without any modifications as this will not impact cleanups, cDNA recovery or sequencing results
Low/no cDNA yield	• Low quality input material	• Reduce fraction of dead cells and debris following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact tissue and avoid freeze-thaw cycles
	• Premature nuclei lysis	• Optimize lysis time for specific cell and tissue type
	• Overly dilute nuclei suspension	• Concentrate nuclei suspension to achieve target concentration of 700 – 1200 nuclei/µl
	• Aggregation of nuclei and/or possible clog during partitioning • Wash/resuspension buffer contains RT inhibitors	• Confirm the use of the correct Nuclei Wash and Resuspension Buffer (1x PBS + 1% BSA + 0.2U/µl RNase Inhibitor) to reduce nuclear aggregation • Nuclei suspensions should always be kept on ice and incubation time be kept to a minimum (<30 min) • Use regular-bore pipette tips during final resuspension of nuclei • Filter nuclei suspension with the appropriate strainer • Nuclei combined with the Single Cell Master Mix should be gently pipette mixed 5 – 10 times with a regular-bore pipette tip and samples immediately loaded with the same pipette tip into the chip
	• Counting inaccuracy results in a low number of cycles during cDNA amplification	• Perform 3 – 4 reproducible counts of the final nuclei suspension (where the standard deviation of these counts is <25%) • Increase the recommended number of cycles during cDNA Amplification by 1 – 2 cycles • Run sample aliquot undiluted on the Bioanalyzer for QC
Low final library yield	• Low number of cycles during SI-PCR	• Increase the recommended number of cycles during SI-PCR by 1 – 2 cycles

5.3. Troubleshooting Data Analysis

Problem	Possible Reason	Solution
Low (<50%) "Fraction Reads in Cells"	• High fraction of ambient RNA in nuclei suspension • Low cell viability prior to lysis	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Add 1 – 2 additional wash steps when preparing the input material • Remove myelin and other impurities with myelin removal beads and/or density gradient centrifugation (post lysis) • Use flow cytometry to sort sample
Low percentage of reads aligned to the reference	• Poor sample quality	• Optimize cell/tissue dissociation protocol for improved sample quality
	• Poorly annotated reference	• Re-run Cell Ranger™ pipeline with custom reference. Visit the 10x Genomics® Support site for more details
High multiplet rate	• Sticky nuclei • High nuclei concentration (> 5000 nuclei/µl) • Use of wide-bore pipette tips during nuclei resuspension • Prolonged incubation of nuclei suspension on ice prior to chip loading • Combined lysis of multiple cell types	• Use regular-bore pipette tips during final resuspension of nuclei • Reduce nuclei concentration to <5000 nuclei/µl • Reduce nuclei processing time • If running mixed species samples, lyse each sample individually followed by mixing both nuclei suspensions just prior to chip loading
Low library complexity (low number of genes/UMI's per cell)	• Low cDNA yield • Low quality input material	• Reduce fraction of dead cells and debris in the input material following Demonstrated Protocol *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093) • Obtain intact tissue and avoid freeze-thaw of cycles • Optimize cell/tissue dissociation protocol for improved sample quality • Optimize lysis time for specific cell and tissue type • Filter nuclei suspension with the appropriate strainer • Remove myelin and other impurities with myelin removal beads and/or density gradient centrifugation • Use flow cytometry to sort sample

5.4. References

- *Removal of Dead Cells from Single Cell Suspensions for Single Cell RNA Sequencing* (Document CG000093)
- *Guidelines for Accurate Target Cell Counts Using 10x Genomics® Single Cell Solutions* (Document CG000091)
- *Single Cell Suspensions from Cultured Cell Lines for Single Cell RNA Sequencing* (Document CG00054)
- *Fresh Frozen Human Peripheral Blood Mononuclear Cells for Single Cell RNA Sequencing* (Document CG00039)
- *Fresh Frozen Human-Mouse Cell Line Mixtures for Single Cell RNA Sequencing* (Document CG00014)
- *Dissociation of Mouse Embryonic Neural Tissue for Single Cell RNA Sequencing* (Document CG00055)