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Nuclei Prep from Frozen Mouse Brain Tissues with Optional Sucrose Column for single nuclei RNA-seq

Greggory A Perry¹, Sandra L Daigle², Paul Gabriel¹, Diane Luo¹,
Jessica Grassmann¹, William F F Flynn¹, Elise T Courtois¹, Paul Robson¹

¹Single Cell Biology Lab, The Jackson Laboratory, USA;

²Single cell Biology Lab, The Jackson Laboratory

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JAX Single Cell Biology

Tech. support email: scblservice@jax.org

Greggory Perry

This protocol has only been tested on a limited number of tissues which includes human and mouse brain frozen tissue. Before using this protocol in production on your tissue of interest, run a pilot experiment to determine optimal dissociation of your tissue.

This SOP aims to extract nuclei from unfixed snap-frozen mouse prefrontal cortex, hippocampus, and Striatum tissues (~3-15mg). It was optimized and tested on mice ranging in age groups from 3, 6, 14, and 15-month-old mice on different diets, including high-fat (45%) and regular chow (6%). Other differences included sex, strain, and genotype. These preps resulted in a minimum of debris for downstream snRNAseq HT assays on the 10X Genomics chromium X controller (HT, High Throughput chips).

This protocol was also used to perform snRNAseq assays from human glioblastoma snap frozen tissues. Follow BSL-2 guidelines when working with human tissues. These preps resulted in a minimum of debris for downstream snRNAseq ST assays on the 10X Genomics chromium X controller (ST, Standard Throughput chips).

This protocol enables to process up to 8 samples simultaneously.

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<https://www.jax.org/>

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nuclei, frozen, brain, single nuclei RNAseq, 10x genomics, luna, Syto Select RNA, hoechst

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A. Use wide-bore pipette tips to avoid damage when handling tissue or nuclei, and pipette slowly.

B. Keep tissue on dry ice until ready to start gentleMACS.

C. Miltenyi gentleMACS Recommendations.

Tissue 1-4 mg - 4C_nuclei_1

Tissue 5-7 mg - cus_4C_nuclei_5

Tissue 7-15 mg - cus_4C_nuclei_10

The "cus" protocols are custom SOP from Miltenyi. It adds 5 or 10 minutes of mixing at 20 RPM after the Miltenyi 5-minute disassociation SOP 4C_nuclei.

* Optimization may be needed depending on tissue type, size, and condition.

D. "To sucrose" or "not to sucrose".

If initial sample size is < 5 mg, it is recommended to wash nuclei without using a sucrose gradient (Step 3). If initial sample size is > 5 mg, it is recommended to purify nuclei using a sucrose gradient (Step 2).

E. Sucrose column recommendations.

After the sucrose cleanup, a tissue less than 3-5 mg may have a very low nuclei yield. It is recommended to test this first on a representative sample to ensure precious samples are not lost.

Hoechst/Syto RNA Select Staining Guidelines

1. Extracellular debris should be minimal after the nuclei have been passed through the sucrose column.
2. Extracellular debris will be present in a much greater quantity if a sucrose column is not used. Debris can interfere with downstream 10x operations by introducing clogs and background ambient RNA.
3. Nuclei should be intact, with minimal blebbing and clumping. Blebbing of nuclei will make them more prone to clumping, which can introduce clogs when loading the 10x chip.
4. The Hoechst and Syto Select staining should be uniform through the nuclei, but the Syto staining usually has more variation.

If working with human tissue, follow all institutional rules and guidelines.

Nuclei isolation

Miltenyi Nuclei Extraction Buffer: 130-128-024 (Miltenyi)

Miltenyi C Tubes: 130-093-237 (Miltenyi)

MACS BSA Stock Solution: 130-091-376 (Miltenyi)

Fisher Scientific DPBS: 14190144 (Thermo Fisher Scientific)

Millipore Sigma ROCHE Protector RNase Inhibitor: 3335399001 (Millipore)

Thomas Scientific Eppendorf™ DNA LoBind 1.5 mL Microcentrifuge Tubes: 13-698-791 (Thomas Scientific)

Fisher Scientific Lobind Eppendorf 2.0mL tubes: 13-698-792 (Thermo Fisher Scientific)
 pluriSelect -BOApluriStrainer Mini 100 µm: 43-10100-50 (pluriSelect)
 pluriSelect -BOApluriStrainer Mini 40 µm: 43-10040-50 (pluriSelect)

Buffers and stocks			
Reagents needed when using sucrose column			
Reagent	Stock Concentration	Final concentration	volume X1
Nuclei Extraction Buffer			
Miltenyi Nuclei Extraction Buffer	n.a.	n.a.	1592
RNAse inhibitor	40 U/µL	0.2 U/µL	8
Sucrose Solution (50 ml Stock Solution)			
Reagent	Stock Concentration	Final concentration	volume X1 (µl)
Sucrose	n.a.	1.8M	30.8 g
Mg(Ac) ₂	1M	3 mM	150 µl
Tris-HCl pH7.4	1M	10 mM	500 µl
H ₂ O	n.a.	n.a.	Q.S. to 50 ml
Sucrose Column			
Sucrose Solution	1.8M	1.8M	750 µl
DTT	1.3M	1mM	0.58 µl
Nuclei Suspension Buffer (750 µl per reaction)			
Reagent	Stock Concentration	Final concentration	volume X1 (µl)
PBS	n.a.	1x	580.67
BSA	10%	2%	150.00
1 mM DTT	1.3 M	1 mM	0.58
RNAse inhibitor	40 U/µl	1 U/µl	18.75
		Total	750.00
Reagents needed when omitting sucrose column			
Reagent	Stock Concentration	Final concentration	volume X1 (µl)
Nuclei Extraction Buffer			
Miltenyi Nuclei Extraction Buffer	n.a.	n.a.	1592
RNAse inhibitor	40 U/µL	0.2 U/µL	8
Nuclei Suspension Buffer (750 µl per reaction)			
Reagent	Stock Concentration	Final concentration	volume X1 (µl)
PBS	n.a.	1x	967.78
BSA	10%	2%	250
1 mM DTT	1.3 M	1 mM	0.97
RNAse inhibitor	40 U/µl	1 U/µl	31.25
		Total	1250

Cell Counting

Acridine, Orange/Propidium Iodide Stain: F23001(New England BioGroup, LLC)

Luna Slides:

Ultra-low Fluorescent Slides: Fluorescent and Brightfield Mode - Choose appropriate size)(New England BioGroup, LLC)

Countess II FL Slides:

Countess™ Cell Counting Chamber Slides: C10312(Thermo Fisher Scientific)

Cellometer K2 Slides:

Cellometer SD100 Counting Chamber (Nexcelom Bioscience)

Staining

PBS: 14190-144(Gibco)

BSA: 130-091-376 (Miltenyi)

Syto RNaselect stain: S32703 (Fisher)

Invitrogen Hoechst 33342, TriH: H3570 (Thermo Fisher Scientific)

The Miltenyi gentleMACS should equilibrate at 4°C for 24 hours before use (cold room or glass door fridge) and should not be used for 24 hours after being moved back to room temperature (It can stay at 4°C).

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This protocol has only been tested on a limited number of tissues which includes human and mouse brain frozen tissue. Before using this protocol in production on your tissue of interest, run a pilot experiment to determine optimal dissociation of your tissue.

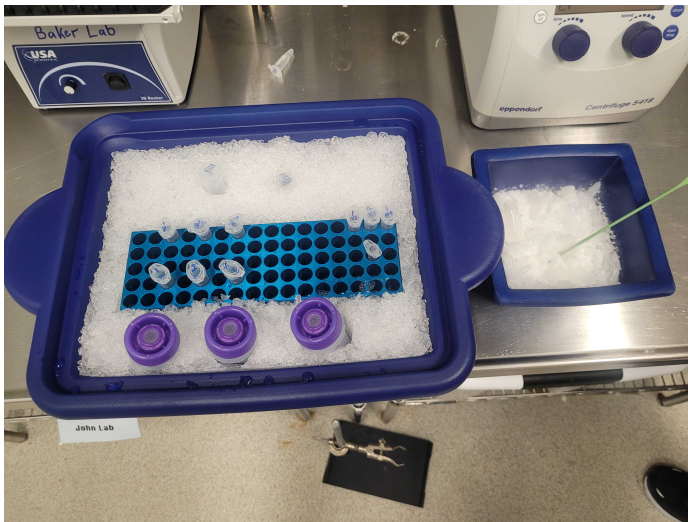
- A. Pre-cool centrifuge, buffers, and consumables with sample contact (e.g., gentleMACS C Tube and Strainers) at 4 °C.
- B. Per extraction, add 0.2U/ul RNase inhibitor to one 1.6 ml aliquot of Miltenyi **Nuclei Extraction Buffer (8µl of 40U/uL RNase inhibitor)** and keep on ice.
- C. Per extraction, add 1 mM DTT to one 750 µl aliquot of **Sucrose Solution (0.58µl 1.3M DTT) in a 2 ml tube** and keep on ice.
- D. Per extraction, prepare 750ul **Nuclei Suspension Buffer** per sample.



Procedure- gentleMAC

1

Miltenyi gentleMACS Procedure for Tissue Lysis and nuclei extraction

Please see the **Guidelines & Warnings** section before beginning. This protocol was optimized to process up to 8 samples simultaneously. Some practice is required to process 8 samples at once. Work fast but cautiously..



- 1.1 Add  **1250 μ L ice-cold Nuclei Extraction Buffer**  **On ice** to each pre-cooled gentleMACS C Tube.
- 1.2 Transfer frozen tissue piece(s) into the gentleMACS C Tube containing lysis buffer and directly proceed with the following steps until samples are dissociated.

Note: Do not let frozen samples thaw before dissociation, as endogenous RNase might degrade RNA.
- 1.3 Close gentleMACS C Tube and place it on the gentleMACS Dissociator.

Be sure the cap is closed completely.



gentleMACS
gentleMACS™ Octo Dissociator with Heaters
Miltenyi Biotec 130-096-427 [↗](#)

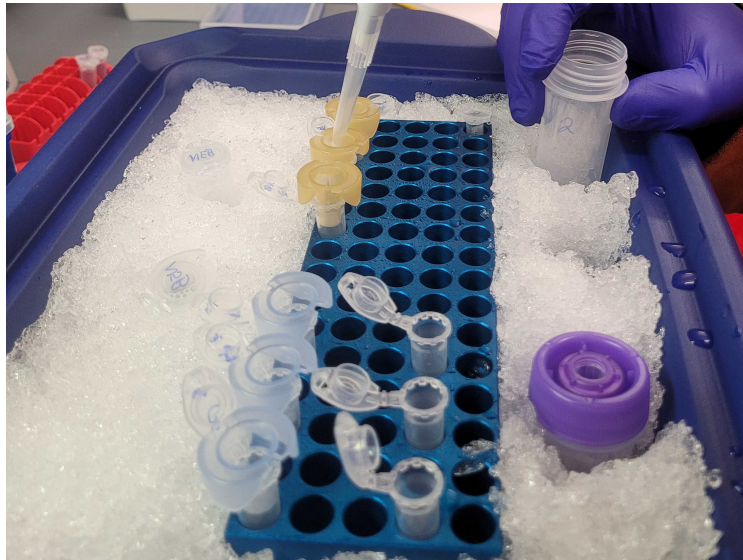
- 1.4 Run gentleMACS Program based on tissue size on the gentleMACS Dissociator at **4 °C**.

See note C under Guidelines & Warnings.

- 1.5 A quick spin in a swinging bucket at **500 x g, 4 °C** for 10-15 sec may be necessary.



- 1.6 Pipette mix with wide bore tip and filter through pre-wet 100µm filter into a cold 2mL tube.
- Pre-wet filter with **Nuclei Extraction Buffer**.
 - Add **250 µL Nuclei Extraction Buffer** to rinse the filter.
 - Spin in a benchtop centrifuge for 5-10 seconds to remove foam if necessary.



1.7 Spin down nuclei, 500 x g , 4°C , $00:05:00$ in a swinging bucket.

5m

1.8 Remove supernatant and resuspend nuclei in
500 μL Nuclei Suspension Buffer

If the pellet is loose, leave **50 μL Nuclei Extraction Buffer** in the tube.

See the Guidelines & Warning section D to decide to proceed to step 2 (sucrose column) or 3 (no sucrose column) next.

Sucrose Column

2 Sucrose Column Purification

2.1 In a 2mL microcentrifuge tube with **750 μL Sucrose Buffer**, layer
500 μL Sample on top, forming two layers.

2.2 Spindown, 16000 x g , 4°C , $00:15:00$ in a fixed angle rotor.

15m

- 2.3 After centrifugation, purified nuclei should be visible as a pellet on the bottom of the tube, and white matter/debris should float midway in the 2 ml tube.



- 2.4 Carefully pour off and blot the tube or aspirate the debris with the supernatant. Remove as much supernatant as possible without disturbing the nuclei pellet.
- 2.5 Resuspend the nuclei pellet in 50-150µl Nuclei Suspension Buffer and let sit on ice without pipetting for 3-5 minutes to loosen the pellet.

- 2.6 Pre-wet 40µm filter with **Nuclei Suspension Buffer**.

Gently pipette mix 10-15x with wide bore tip and filter through pre-wet 40µm filter into new cold 1.5mL tube.

Be careful not to wash down sucrose waste from the tube walls.


Proceed to Step 4 and 5.

Wash without Sucrose Column

3

Washing nuclei without the use of a sucrose column

- 3.1 Add **500 µL Nuclei Suspension Buffer** and gently pipet mix 5-10x using wide bore tip to suspend nuclei.

3.2 Centrifuge in a swinging bucket  **350 x g, 4°C, 00:05:00** .

3.3 Remove supernatant, being careful not to disturb the nuclei pellet.

3.4 Repeat Steps 3.1 - 3.3 for a total of 2 washes.

3.5 Resuspend the nuclei pellet in 50-150µl **Nuclei Suspension Buffer** and let sit on ice without pipetting for 3-5 minutes to loosen the pellet.

3.6 Pre-wet 40µm filter with **Nuclei Suspension Buffer**.

Gently pipette mix 10-15x with wide bore tip and filter through a pre-wet 40µm filter into new cold 1.5mL tube.
Proceed to step 4 and 5.

Nuclei counting

4 Nuclei counting with Trypan Blue / AOPI stain

Obtain nuclei in suspension buffer from steps 2 or 3.

Proceed to Step 4.1 if using Luna FX7 cell counter and AOPI stain to count nuclei.

Proceed to Step 4.2 if using Countess FL II cell counter and Trypan Blue stain to count nuclei.

Proceed to Step 4.3 if using Nexcelom Cellometer K2 and AOPI stain to count nuclei.

Note: Trypan Blue staining is useful to visualize debris and nuclei qualitatively. The counts using Trypan include both nuclei and debris. AOPI is used to count only nuclei. By comparing Trypan and AOPI counts, one can quantitatively assess the amount of debris present in samples preps.

4.1 Nuclei counting with LunaFX7

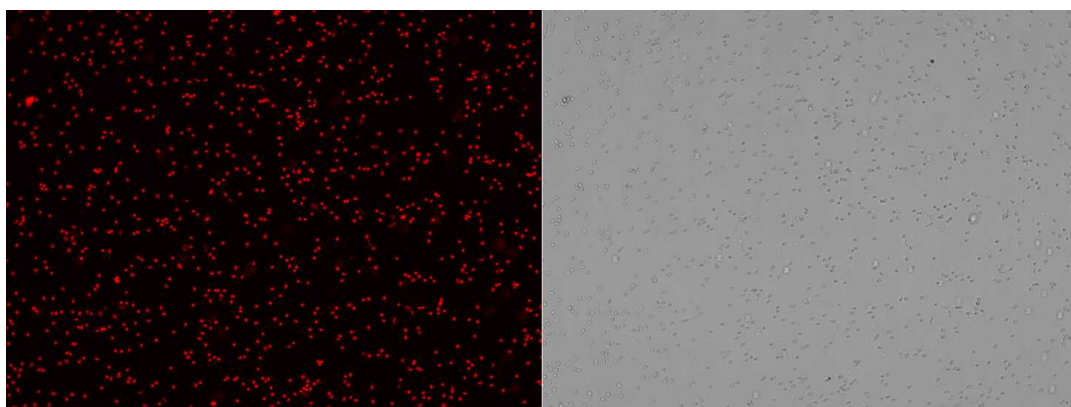
A. Add 1uL AOPI stain and 9uL suspended nuclei to a .5 ml microcentrifuge tube or PCR strip tube and mix gently by pipetting 5x.

Examples:

1ul AOPI/9ul sample

1ul AOPI/4.5ul sample/4.5 ul suspension buffer

- B. Turn on Luna FX7 cell counter.
Select "fluorescence cell counting" and "cell lines & primary cells" with the touch screen menu.
- C. Select the settings tab and choose the correct slide and wells being counted.
- D. Load preferred protocol for fluorescence cell counting.
- E. Load preferred protocol for fluorescence cell counting.
- F. Select Count, insert the slide into the LunaFX7, and pre Count on the right-hand menu.
- G. Record desired data after Count is finished using the save and print command, which allows renaming of the sample, or use quick save, which auto-generated a name.
- H. Below is an example of Luna FX7 images.



Left image shows "dead" cells or nuclei (red, nuclei stained with PI). Right image shows the brightfield for the same field.

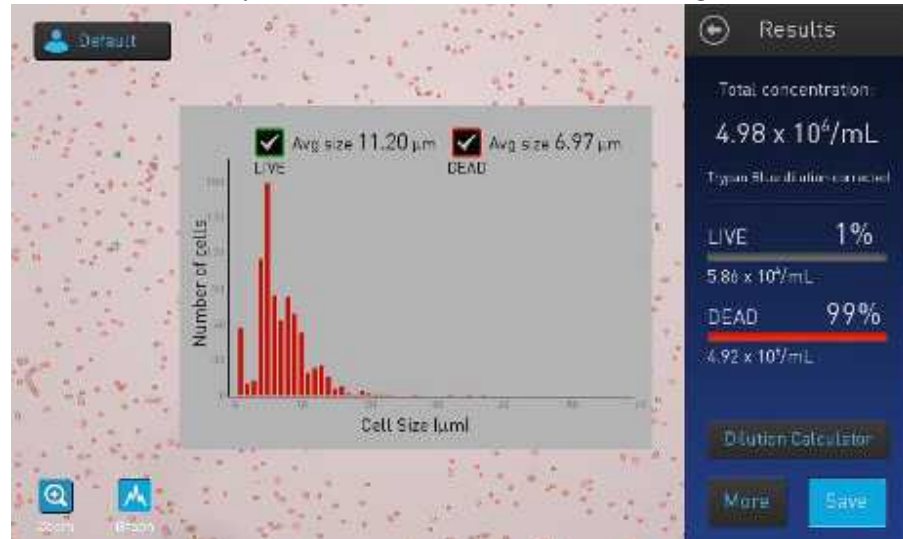
4.2 Nuclei counting with Countess FL II

- A. Add 6 uL Trypan Blue stain 0.4% and 6 uL nuclei suspension to 0.2 mL PCR strip tube. Mix gently by pipetting 5-10x.
- B. Load 10 uL into Countess cell counting chamber slide.
- C. Visually assess nuclei sample in slide using bench top microscope, 20x magnification. Note debris present, if any, and overall nuclei shape/integrity.
- D. Load slide into Countess FL II. Adjust brightness and focus if necessary. Click

"Count".

E. Once counting has been completed, record nuclei concentration, viability, and size. Save data with correct date and sample name.

F. Below is an example of Countess FL II readout showing "dead" cells, or nuclei.



4.3 Nuclei counting with Nexcelom Cellometer K2

A. Add 11 µL AOPI stain and 11 µL nuclei suspension to 0.2 mL PCR strip tube. Mix gently by pipetting 5-10x.

B. Load 20 µL into Cellometer SD100 slide.

C. Record nuclei concentration, viability, and size.

Nuclei quality QC: Hoechst and SYTO RNASelect staining of nuclei

5 Hoechst and SYTO RNASelect staining of nuclei

Keep all stains and dilutions on ice in the dark **4 °C**.

Choose 5.1 for Leica DM4 B or 5.2 for PerkinElmer Opera Phenix.

5.1 Nuclei QC with Leica DM4 B

20m

Imaging:

Microscope - Leica DM4 B
Camera - Leica DCF7000 GT

Exposure time:

Hoechst - 45 ms, gain 5.5

SYTO RNASelect - 800 ms, gain 3.4

Brightfield - 30 ms, gain 8.8

Magnification - 40X

- The exposure time and gain may need to be adjusted depending on the microscope used.

A. Make a 1:80 dilution of Hoechst stain (1ul into 79ul PBS)

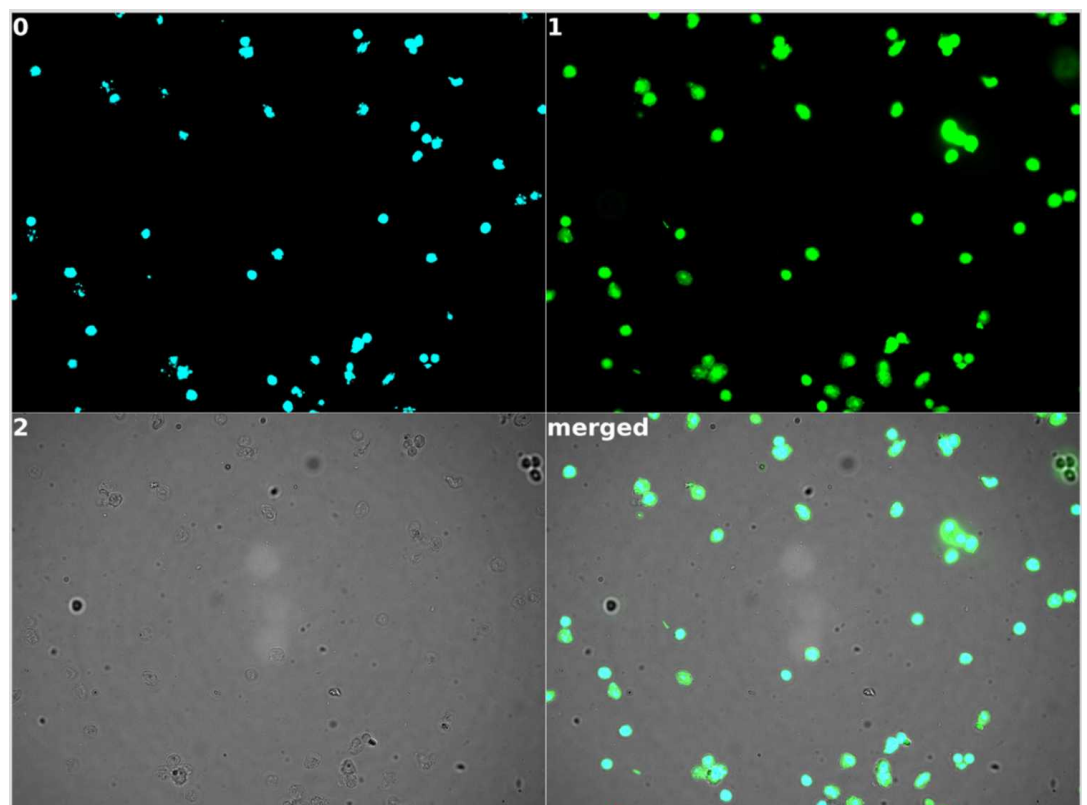
B. Make a 1:500 dilution of SYTO RNASelect stain (1ul into 499ul PBS)

C. Mix well and aliquot 18ul of nuclei suspension into a new microcentrifuge tube. Dilute if needed at this point, but the total volume should be 18ul.

D. Add 1ul 1:80 Hoechst stain and 1ul 1:500 SYTO RNASelect stain to 18ul suspended nuclei and mix gently 5x pipetting. Immediately place the mixture in a light-blocking box and place it at **4°C, 00:20:00**.

Expected results of nuclei put through a sucrose column are shown below.

See guidelines for further details.



0- Hoechst Stain
1- SYTO RNASelect Stain
2- Brightfield
merged - an overlay of all images


5.2 Nuclei QC with Opera Phenix confocal microscope with 40x water objective. 20m

A. Dilute SYTO RNASelect stain 1:1000 and dilute Hoechst 1:100 in DPBS. Keep dye dilutions on ice and protected from light.

B. Place a 384-well plate (PerkinElmer CellCarrier Ultra) on ice.

C. Add 20 μ L of nuclei suspension to a 0.2 mL strip tube.

D. Add 1 μ L each of diluted SYTO RNASelect and Hoechst stains into tube containing nuclei suspension. Pipet mix 5-10x.

E. Transfer contents of strip tube into 384-well plate. Incubate 384-well plate at  **4°C, 00:20:00** protected from light.

F. Image 384-well plate using the following parameters, ensuring that no channels are over/underexposed:

Plate Type: 384 PerkinElmer CellCarrier Ultra

Autofocus: Two Peak (Default)

Objective: 40x Water, NA 1.1

Opt. Mode: Confocal

Binning: 2

Channel Selection:

Brightfield

Exposure Time = 200 ms

Power = 100%

SYTO RNA Select

Exposure Time = 100 ms

Power = 50%

HOECHST 33342

Exposure Time = 100 ms

Power = 50%

Layout Selection (Well)

Number of Fields = 49

Overlap = 5%

Layout Selection (Stack)

First Plane = -1.0 μ m

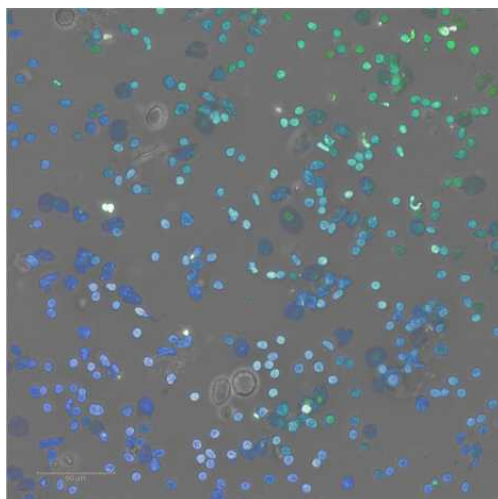
Number of Planes = 6

Distance = 3.0 μ m

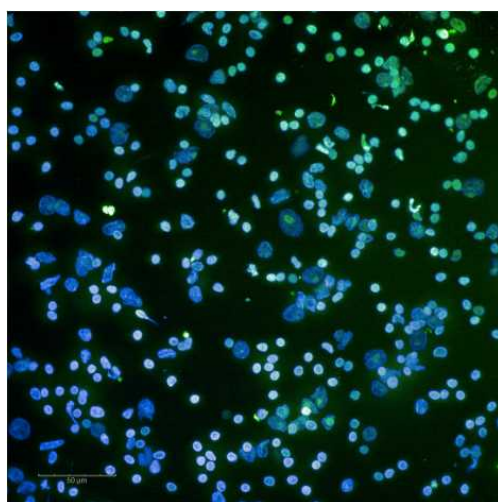
Last Plane = 14.0 μ m

Overall Height = 15.0 μ m

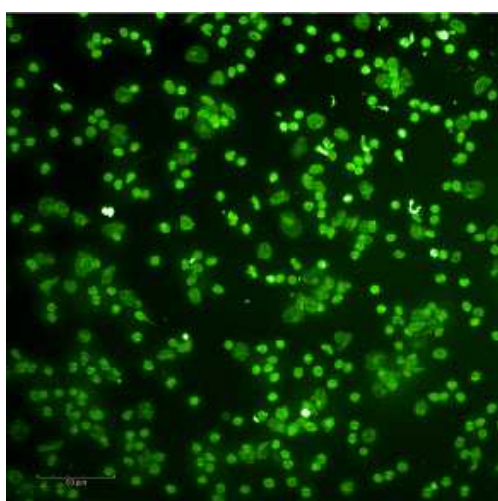
G. Example nuclei QC images are below.



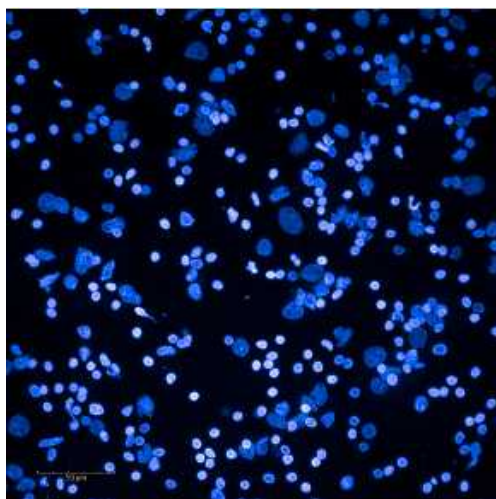
Brightfield + Hoechst + SYTO RNASelect



Hoechst + SYTO RNASelect



SYTO RNASelect



Hoechst

Loading nuclei on 10x Genomics chip

6 10x Genomics downstream applications

Follow 10x recommendations for your specific needs.

For a 10x standard chip (ST) and High Throughput (HT) chip, calculate the number of nuclei that needs to be loaded for each channel, and according to the experimental design.

Loading examples:

ST - Targeting 6,000 nuclei

- load 12,000 to recover 12,000 taking into account a 50% capture rate.

HT - Targeting 20,000 nuclei

- load 33,300 to recover 20,000 taking into account a 60% capture rate.

For more detailed guidelines visit <https://www.10xgenomics.com/> for more details.