tapestri

Nuclei Extraction From Frozen Tissue For Single-Nuclei DNA Sequencing

User Guide

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Introduction

Single-cell analysis provides a unique opportunity to better understand cellular heterogeneity that governs numerous biological processes including tissue development, disease progression and drug response. In particular DNA sequencing at the single-cell level holds great promise in gaining insights into the genetic variability underlying complex human diseases, such as cancer, and advancing the field of precision medicine[1]. For that reason, Mission Bio developed the Tapestri Platform that uniquely provides a targeted, automated and scalable approach to profile single nucleotide variants (SNVs) and indel mutations across thousands of cells at cell-to-cell level.

Standard single-cell sequencing strategies demand cell suspensions that consist of thousands of individual cells that are viable and intact. This demand, however, excludes a plethora of clinically valuable and widely accessible sample types from being considered as they often do not meet those input material requirements (e.g. samples banked as frozen tissue sections)[2]. Protocols to efficiently isolate individual nuclei from challenging sample types such as frozen tissue sections and methods to profile them on a nuclei-to-nuclei level are therefore essential.

To address this deficit, we have developed a rapid and simple to use nuclei extraction protocol to isolate nuclei from frozen tissues or cells. The method presented in this guide is an adaptation of the protocol described by Vindelov[3]. The method uses detergent-trypsin for nuclei preparation and is broadly used in clinical laboratories for DNA analysis. The protocol's low cost, short time and relatively low sample input requirements (<8 mm3) makes it particularly attractive for scientists in the clinical and translational research space. As an alternative, we present a customer-developed protocol for frozen tissues that is an adaptation of the protocol described by Martelotto[4]. This method utilizes a commercially available lysis buffer and mechanical dissociation for nuclei preparation and is widely used by researchers for single nuclei experiments using droplet-based methods. This protocol has been shown to work successfully with fresh and frozen cryopreserved cells and cell lines, as well as tissue sections from lymphomas, pancreas, brain, breast, lymphoma and xenografts.

About This Guide

Here we introduce two rapid methods with step by step instructions for nuclei preparation from frozen tissues.

The expected capture rate of intact cells in Mission Bio's generated droplet technology is typically 5-10% (5,000-10,000 cells). As nuclei exhibit differential flow kinetics within microfluidics channels compared to much larger cells, a typical nuclei capture will result in fewer organelles being captured over the 5 min encapsulation process. Commonly one can expect a capture in the 2,000-4,000 range for a high quality nuclei prep on Tapestri.

Nuclei Handling Guidelines

The steps provided in this protocol are applicable to snap frozen or OCT-embedded tissue fragments, either stored at -80 °C or liquid nitrogen, as well as frozen tissue sections from brain, breast, colon, lung, kidney, liver and prostate. The nuclei extraction protocols outlined in this guide require a minimum starting material of 30 mg of tissue. It is recommended that you start with 50-100 mg of material when optimizing the protocol for a tissue type for the very first time. In the case of frozen tissue sections, 50-100 µm thickness is recommended. Thicker sections increase the absolute number of intact nuclei compared to thinner sections and minimize the number of partial nuclei generated during tissue sectioning. When the tissue sample is comprised of 70% nucleated cells, from 3 x 3 x 3 mm of frozen tissue section, between 5×10^5 - 7×10^6 intact nuclei may be recovered. Refer to ***Appendix A*** for information on how to calculate the section's tissue volume, based on its thickness. Please note that the amount of OCT in each tissue section varies and needs to be taken into consideration when estimating the tissue weight. Stromal tissue in general, has a significantly lower density of nucleated cells than most of the epithelial or tumor tissues. Thus, when extracting nuclei from tumor samples, the ratio of stromal:tumoral cells may also be considered as a factor impacting overall nuclei yield. In addition, cell populations can differ from specimen to specimen (necrotic, apoptotic, inflammatory, stromal, fibrotic cells), especially in tumor tissues. Hence, the use of a representative H&E stained tissue section from the specimen-matched tissue is recommended to evaluate the tissue area, types of cells present and percentage of nucleated cells.

Wash the harvested tissue in a petri-dish with cold PBS to remove blood and absorb excess blood /solution from the surface with a laboratory wipe to limit ice crystal formation during freezing. Tissues should be stored in a cryovial in liquid nitrogen for best results. Tissues can be stored short-term (1-2 days) at -80˚C if needed. Once tissues are removed from liquid nitrogen, tissue should be kept at -80˚C or on dry ice until use.

Different tumor types will require optimization of the current protocol for lysis incubation time, number of wash steps, cell strainer mesh size, centrifugation speed, re-suspension volumes and quantitation methods. Mission Bio strongly recommends processing the single nuclei suspension on the Tapestri Platform immediately after successful extraction. Storage of the nuclei suspension overnight at 4°C is not recommended.

Please contact support@missionbio.com for additional support.

Materials

Required Consumable Reagents

Component Name	Suggested Supplier (Part Number)
5 mL DNA LoBind Eppendorf tubes*	Eppendorf (30108310) or equivalent
50 mL tubes	VWR (89004-364) or equivalent
1.5 mL DNA LoBind Eppendorf tubes*	Eppendorf (022431021) or equivalent
15 mL tubes	Thermo Fisher Scientific (339651) or equivalent
200 µL, 10 µL, 1 mL low retention pipette tips.	General lab supply
50 µm CellTrics® cell strainer	Sysmex (04-004-2327)
30 µm CellTrics® cell strainer	Sysmex (04-004-2316)
Hemocytometer	VWR (15170-070) or equivalent
DAPI Solution 1 mg/mL	Thermo Fisher Scientific (62248)
NucBlue™ Live ReadyProbes™ Reagent	Thermo Fisher Scientific (R37605)
Trypsin inhibitor from chicken egg white, Type II-O	Sigma (T9253)
Sodium citrate tribasic dehydrate	Sigma (C8532)
Spermine Tetrahydrochloride	Sigma (S1141)
Tris (Hydroxymethyl) aminomethane	Sigma (252859)
IGEPAL CA-630	Sigma (I8896)
Trypsin-EDTA (0.25%), phenol red	Thermo Fisher Scientific (25200072)
Collagenase	Worthington (CLS-7 LS005332)
Dispase II	Gibco (17105-041)
Ribonuclease A from bovine pancreas, type I-A.	Sigma (R4875-100mg)
1x DPBS, no calcium, no magnesium	Thermo Fisher Scientific (14190-136) or equivalent
SPRUCE: Sterile Disposable Scalpel With Carbon Steel Blade #22 And Plastic Handle	Amazon (B008S2B3D0)
Dry ice in pellets	
Sterile Petri Dishes	VWR (664160)
HCL, Molecular Biology grade	Sigma (H1758)
UltraPure™ BSA (50 mg/mL)	Thermo Fisher Scientific, AM2618
Dounce Tissue Grinder Set (for mechanical dissociation protocol)	Sigma (D8938-1SET)
Nuclei EZ lysis buffer (for mechanical dissociation protocol)	MilliporeSigma / Sigma Aldrich (N3408)

NOTE ***The use of DNA LoBind tubes is strongly recommended to increase the recovery rate of nuclei. This tube fits regular 15 mL centrifuge adapters.***

Optional Consumable Reagents

Component Name	Suggested Supplier (Part Number)
Phalloidin iFluor-555	Abcam, ab176756
WGA -Alexa Fluor 488	Thermo Fisher Scientific, W11261

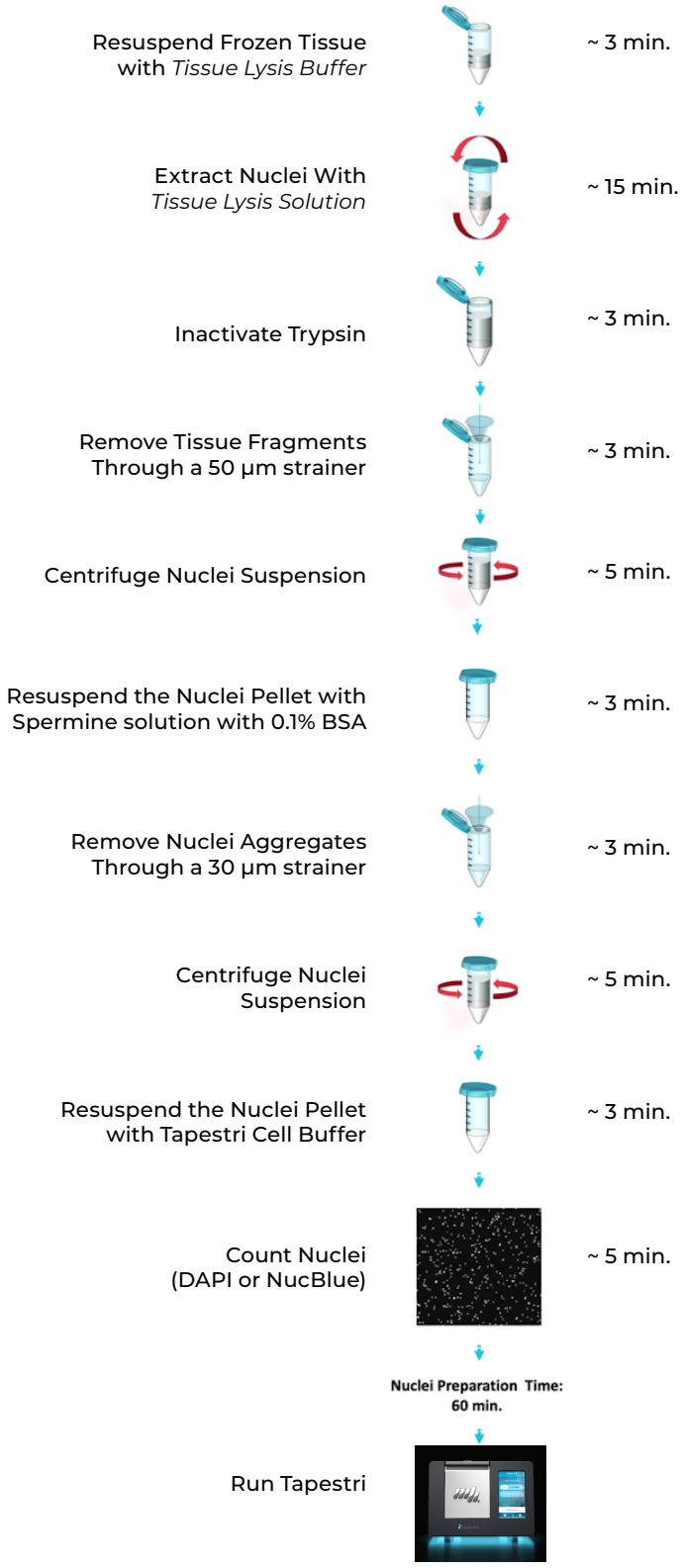
NOTE ***Reagents listed in above table are used for optimizing the protocol (see Appendix B).***

Required Benchtop Equipment

Required Equipment	Suggested Supplier (Part Number)
pH meter	-
Pipettes, 1 µl – 1000 µl	Mettler-Toledo, Rainin Pipettes or equivalent
Refrigerated Centrifuge (15 mL)	Beckman Coulter (B08895) or equivalent
Tube Vortexer	Thermo Fisher Scientific (88880017TS) or equivalent
Fluorescent microscope EVOS FL	Life Technologies or equivalent
HulaMixer Sample Mixer	Thermo Fisher Scientific (15920D) or equivalent

Enzymatic Dissociation Protocol

Protocol Overview



Enzymatic Dissociation Protocol

1. Prepare Stock and Working Solutions

1. Prepare Stock and Working Solutions

1.1 Prepare Stock Solutions according to the following table:

Reagent	Stock Preparation	Final Concentration of Stock Solution
Sodium citrate tribasic dihydrate	Dissolve 14.70 g in 50 mL ddH2O	1 M
Spermine Tetrahydrochloride	Dissolve 8.70 g in 50 mL ddH2O	0.5 M
Tris (Hydroxymethyl) aminomethane	Dissolve 3.03 g in 50 mL ddH2O	0.5 M
IGEPAL CA-630	Dilute 1 mL in 9 mL ddH2O	10%
HCl	Dilute 4.175 mL of 36% HCL in 45.825 mL ddH2O	1 M

IMPORTANT ***Store all solutions at room temperature for up to 2 months.***

1.2 Prepare **Spermine Solution (100 mL)** according to the following table:

Reagent	Stock Solution	Final Concentration	Volume for 100 mL
Sodium citrate tribasic dihydrate	1 M	3.4 mM	340 µL
Spermine Tetrahydrochloride	0.5 M	1.5 mM	300 µL
Tris (Hydroxymethyl) aminomethane	0.5 M	0.5 mM	100 µL
IGEPAL CA-630	10%	0.10%	1 mL
Mol. Biol. Grade Water			98.26 mL

IMPORTANT ***Adjust pH to 7.6. Use a calibrated pH meter and adjust pH using 1 M HCl solution. Store at 4° C for up to 1 month.***

1.3 Prepare **Spermine Solution with 0.1 % BSA (60 mL)** according to following table:

Reagent	Volume for 60 mL
Spermine Solution	58.8 mL
UltraPure™ BSA (50 mg/mL)	1.2 mL

1.4 Prepare **Stop Solution (50 mL)** according to the following table:

Reagent	Amount	Volume for 50 mL
Trypsin inhibitor from chicken egg white, Type II-O.	Add 25 mg	
Ribonuclease A from bovine pancreas, Type I-A.	Add 5 mg	
Spermine Solution (pH7.6) with 0.1% BSA		49.8 mL

NOTE ***The use of RNAse A, helps to minimize the nuclei clumping. The use of Stop Solution without RNAse A or with white precipitates, it is not recommended.***

IMPORTANT ***Adjust pH to 7.6. Use a calibrated pH meter and adjust pH using 1 M HCl solution. Store at 4° C for up to 1 month.***

1.5 Prepare **Nuclei Staining Buffer (1 mL)** according to the following table:

Reagent	Amount	Volume for 1mL
Spermine Solution with 0.1% BSA		960 µL
DAPI or NucBlue	4%	40 µL

IMPORTANT ***Protect the Nuclei Staining Solution from light. Store at 4° C.***

Enzymatic Dissociation Protocol

2. Extract Nuclei

2. Extract Nuclei

IMPORTANT

- Incubate all reagents at room temperature for at least 10 min.
- Thaw the enzymes and keep them on ice.
- Prepare a dry ice bucket.
- For each sample to be processed:
  - Pre-chill on dry ice a pair of sterile scalpels.
  - Pre-chill on dry ice one sterile Petri dish.
  - Prepare a 15 mL LoBind tube with a 50 µm CellTrics cell strainer.
  - Prepare a 5 mL LoBind tube with a 30 µm CellTrics cell strainer.
  - Prepare a 1.5 mL LoBind tube for nuclei quantification.

2.1 Prepare fresh Tissue Lysis Solution in a 5 mL LoBind tube as follows:

Reagent	Stock Concentration	Final Concentration	Volume for 2mL
Trypsin-EDTA (0.25%), phenol red	2.5 mg/mL (0.25%)	0.03 mg/mL (0.003%)	24 µL
Collagenase	8 mg/mL	0.1 mg/mL	25 µL
Dispase II	100 mg/mL	0.1 mg/mL	2 µL
Spermine Solution (pH 7.6)			1.949 mL

2.2 Retrieve cryovial containing the tissue fragment/s from -80°C freezer and **keep on dry ice at all times.**

2.3 **Transfer** the fragment of **tissue into the pre-chilled Petri dish** placed on top of the dry ice.

2.4 From the 2 mL of **Tissue Lysis Solution**, pipette **200 µL on top of the tissue fragment.**

2.5 Incubate for **2–3 minutes until the Tissue Lysis Solution** on top of the tissue fragments **freezes.**

NOTE ***The freezing step in Tissue Lysis Solution provides the tissue with a more homogeneous density and as a result increases the efficiency of tissue mincing, enzymatic disaggregation, and nuclei recovery.***

2.6 Using a chilled disposable sterile scalpel, **mince the tissue fragment/s** embedded in Tissue Lysis Solution thoroughly.

2.7 **Remove the Petri dish** (containing the minced tissue and Tissue Lysis Solution) **from dry ice** and bring to room temperature.

2.8 **Continue mincing the tissue** until the Tissue Lysis Solution is completely thawed and the tissue fragments are small. The tissue fragments are considered small if they can flow through a P-1000 tip without clogging.

2.9 Use the remaining **1800 µL of Tissue Lysis Solution** to **rinse and transfer all the tissue fragments** from the Petri dish back to a 5 mL low binding tube.

2.10 Place the tube on a Sample Mixer and **incubate at room temperature for 15 minutes,** rotating at **20 rpm** (or very low speed). Alternatively, the **tube may be gently inverted repeatedly** by hand to keep the tissue in suspension. **Check** the tissue digestion **every 5 min**, as some tissues may be digested in less than 15 minutes. A tissue that is not successfully digested in 15 minutes, likely will not digest with longer incubations.

IMPORTANT ***When extracting nuclei from tissue types that have not been validated by Mission Bio, it is required to optimize the incubation time. Refer to Appendix B for optimization of nuclei extraction.***

2.11 Add **2 mL of Stop Solution**. Mix by inverting the tube gently. Let sit at room temperature for 1 minute.

2.12 **Filter** the nuclei suspension **through a 50 µm cell strainer** and collect the flow in a 15 mL LoBind tube.

2.13 Wash the filter gently with **1 mL** of **Stop Solution twice (this step is critical for final yield).**

NOTE ***Before filtering the nuclei suspension, wash the strainer with 1 mL of Spermine Solution. It helps to break the surface tension of the mesh and minimizes nuclei loss. In addition, when filtering the nuclei suspension, do not pipette the nuclei suspension directly on the strainer mesh. Instead, pipette against the strainer's wall. Pipetting the nuclei suspension directly onto the strainer mesh can dilate the mesh and increase the fraction of nuclei clumps that may flow through.***

2.14 **Centrifuge** the flow through containing the nuclei at **500 x g for 5 min at room temperature.**

NOTE ***Centrifugation speed is critical in preventing formation of nuclei clumps. Increasing centrifugation speed or time will result in increased nuclei clumping and lead to excess amounts of cellular debris. Upon centrifugation completion proceed immediately to step 2.15.***

2.15 Carefully discard the supernatant and **resuspend the nuclei in 1 mL** of **Spermine Solution with 0.1 % BSA.**

2.16 **Filter** the nuclei suspension through a **30 µm cell strainer** and collect the flow through in the 5 mL DNA LoBind tube.

2.17 Wash the filter gently with **1 mL** of **Spermine Solution with 0.1 % BSA twice** and collect the flow through in the same 5 mL DNA LoBind tube from step 2.16 **(this step is critical for final yield).**

2.18 **Centrifuge** the nuclei suspension **at 500 x g for 5 min at room temperature.**

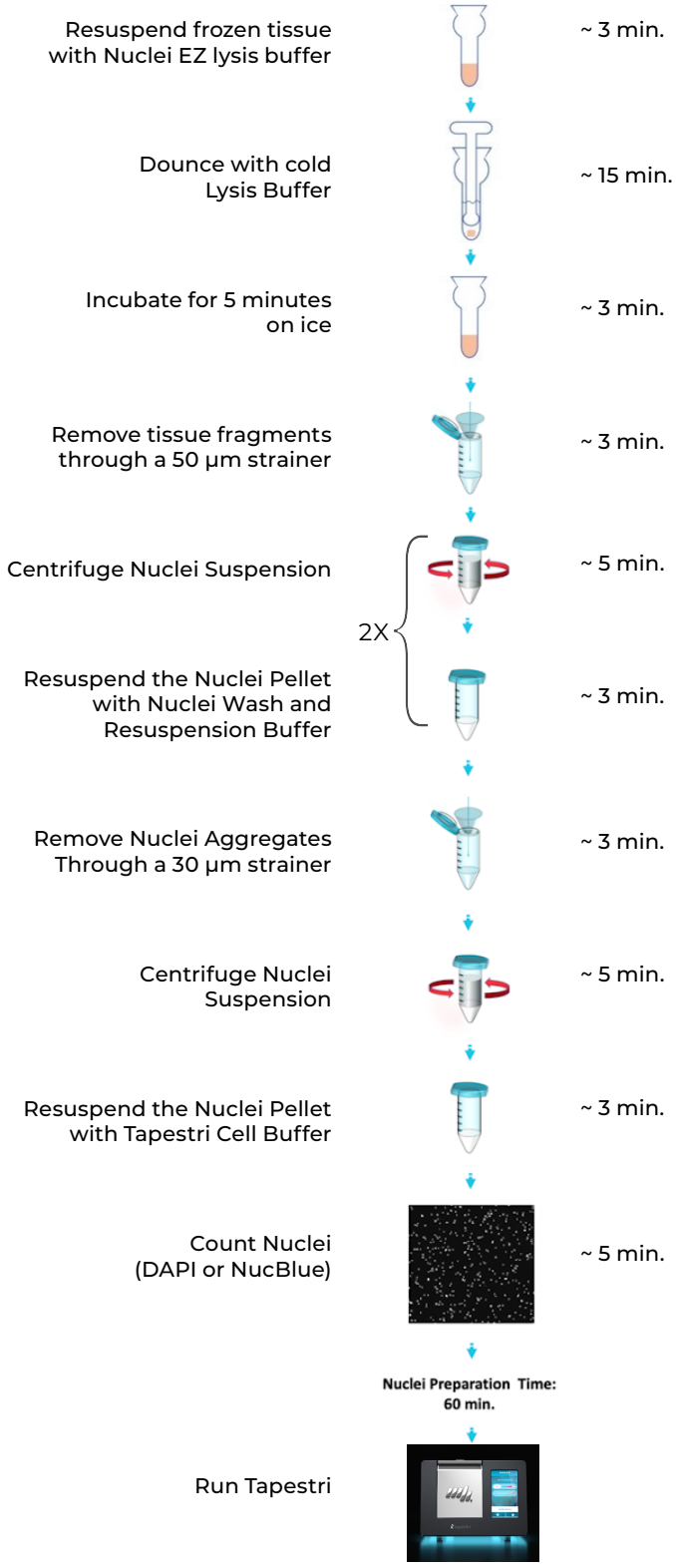
2.19 Carefully discard the supernatant and **resuspend the nuclei in 50-200 µL** of **Mission Bio Cell Buffer (volume should be adjusted based on input material and desired nuclei concentration).**

NOTE ***Mission Bio's Cell Buffer contains density gradient medium. Cells that are resuspended in Cell Buffer are difficult to pellet via centrifugation.***

2.20 **Keep the nuclei suspension on ice** and proceed immediately to ***Section 3 – Count Nuclei***.

Mechanical Dissociation Protocol

Protocol Overview



Mechanical Dissociation Protocol

1. Prepare Working Solutions

1. Prepare Working Solutions

1.1 Aliquot **2 mL of Nuclei EZ Lysis Buffer (Millipore Sigma, Part Number: N3408)** into a 5 mL LoBind tube.

1.2 Prepare **Nuclei Wash and Resuspension Buffer (5 mL)** according to the following table:

Reagent	Volume for 5 mL
UltraPure™ BSA (50 mg/mL)	1 mL
1 X PBS (no Ca/Mg)	4 mL

1.3 Prepare **Nuclei Staining Buffer (1 mL)** according to the following table:

Reagent	Final Concentration	Volume for 1 mL
Nuclei Wash and Resuspension Buffer		1 mL
DAPI or NucBlue	4%	4 µL

IMPORTANT ***All buffers and reagents are kept on ice or chilled at 4 °C. Protect the Nuclei Staining Buffer from light. Store at 4° C.***

Mechanical Dissociation Protocol

2. Extract Nuclei

2. Extract Nuclei

IMPORTANT

- Prepare a dry ice bucket.
- For each sample to be processed:
  - Pre-chill on dry ice a pair of sterile scalpels.
  - Pre-chill on dry ice one sterile Petri dish.
  - Pre-chill dounce 2 mL tube and Pestle A and B (Sigma, Part Number: D8938-1SET) in the fridge.
  - Pre-chill a 2 mL LoBind tube.
  - Prepare a 15 mL LoBind tube with a 50 µm CellTrics cell strainer.
  - Prepare a 5 mL LoBind tube with a 30 µm CellTrics cell strainer.
  - Prepare a 1.5 mL LoBind tube for nuclei quantification.

2.1 Retrieve cryovial containing the tissue fragment/s from -80°C freezer and **keep on dry ice at all times.**

2.2 **Transfer** the fragment of **tissue into the pre-chilled Petri dish** placed on top of the dry ice.

2.3 Using a chilled disposable sterile scalpel, **mince the tissue fragment/s** to small pieces.

2.4 **Transfer** the tissue to a chilled 2 mL Dounce tube and add **500 µL chilled Nuclei EZ Lysis Buffer to the tube.**

2.5 **Dounce with Pestle A** until resistance goes away (~10-20 strokes). **Dounce with Pestle B** for 5-15 strokes.

2.6 **Transfer** the homogenate into a 2 mL LoBind tube and **add 1 mL of chilled Nuclei EZ Lysis Buffer**, mix gently and **incubate on ice for 5 min**. Gently mix with a wide bore tip and repeat 1-2 times during the incubation.

2.7 **Filter** the nuclei suspension **through a 50 µm cell strainer** and collect the flow through in a 15 mL LoBind tube.

2.8 **Centrifuge** the nuclei at **500 x g for 5 min at 4°C**, remove supernatant without disturbing the pellet and **add 500 µL Nuclei Wash and Resuspension Buffer**.

2.9 **Incubate for 5 minutes** without resuspending to allow buffer interchange. After incubation, **add 1 mL of Nuclei Wash and Resuspension Buffer and resuspend the nuclei.**

2.10 **Centrifuge** the nuclei at **500 x g for 5 min at 4°C**, remove supernatant and gently **resuspend nuclei in 1.4 mL Nuclei Wash and Resuspension Buffer**.

2.11 **Filter** the nuclei suspension **through a 30 µm cell strainer** and collect the flow through in the 5 mL DNA LoBind tube.

2.12 **Centrifuge** the nuclei at **500 x g for 5 min at 4°C**, and carefully discard the supernatant and **resuspend the nuclei in 50-200 µL of Mission Bio Cell Buffer (volume should be adjusted based on input material and desired nuclei concentration).**

NOTE ***Mission Bio's Cell Buffer contains density gradient medium. Cells that are resuspended in Cell Buffer are difficult to pellet via centrifugation.***

2.13 **Keep the nuclei suspension on ice** and proceed immediately to ***Section 3 – Count Nuclei***.

Nuclei Dissociation Protocol

3. Count Nuclei

3. Count Nuclei

3.1 **Mix** by gently pipetting up and down.

3.2 **Dilute an aliquot** of the nuclei suspension **1:5 to 1:20** in Nuclei Staining Buffer (10 uL minimum volume).

3.3 Mix by gently pipetting up and down.

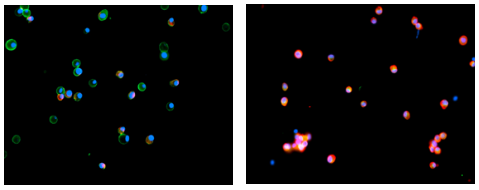
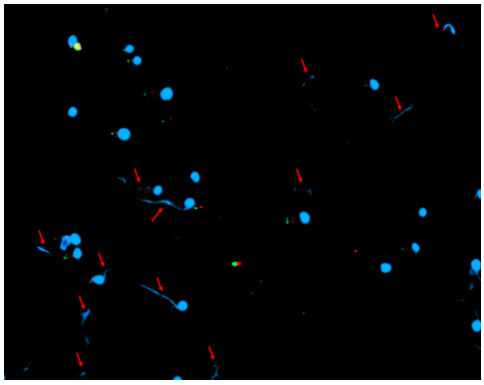
3.4 **Load 10 µl** of the diluted sample on a hemocytometer. Count the nuclei using the DAPI channel on an EVOS FL microscope or equivalent instrument. Follow instrument manufacturer's instructions and hemocytometer good practices (www.hemocytometer.org).

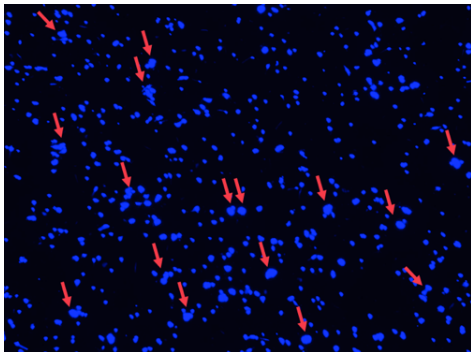
NOTE ***When quantifying the sample in a hemocytometer using DAPI or NucBlue staining, ensure the nuclei suspension contains < 5% clumps (a clump defined as ≥ 3 aggregated nuclei).***

3.5 Dilute sample to a concentration within 3000-4000 nuclei/ul using Mission Bio Cell Buffer. Process nuclei suspension on the Tapestri Platform following the Tapestri Single-Cell DNA Sequencing User Guide (PN 3354).

NOTE ***When diluting the sample, make sure that the volume of Cell Buffer added is = or > 50% to prevent nuclei from sinking/aggregating when loaded onto the Tapestri cartridge.***

Troubleshooting*

Problem	Description & Recommended Action
Under digested nuclei 	**Description:** Depending on the incubation time used for the lysis of samples, the cells can show a strong staining for cytoplasm (RFP, red) or membrane (GFP, green) markers. Strong staining of either marker indicates that the sample is under digested. **Suggestion:** If cell suspension is uniform without clumping (A), proceed with confidence. If clumps are >5% (B), increase the incubation time with Tissue Lysis Solution. Note: A clump is defined as ≥ 3 aggregated nuclei.
Over digested nuclei 	**Description:** Tissues other than used in this present protocol may be more sensitive to the enzymatic dissociation conditions. As a result, nuclei may burst while resuspending the nuclei pellet in Cell Buffer, releasing the DNA and forming an insoluble cloud. Occasionally unfavorable lysis conditions result in broken nuclei, which may release DNA into solution (see red arrows), with no stain for cytoplasm (RFP, red) or membrane (GFP, green) markers. **Suggestion:** Reduce the incubation time in Tissue Lysis Solution. * Please refer to ***Appendix B*** for additional information about the staining patterns.
Inaccurate Nuclei quantification	**Description:** Automatic cell counters are accurate to quantify intact cells, however, nuclei may not be accurately detected. This is due to (1) the light refracting differently in cells than in nuclei and (2) the size of the nuclei falling into the lower limit of detection of the instruments. Also, quantifying nuclei with trypan blue can result in overestimation of nuclei concentration due to presence of tissue debris or precipitated crystals of the dye. **Suggestion:** Use a hemocytometer and a fluorescent microscope to accurately quantify DAPI+ nuclei.

Problem	Description & Recommended Action
Presence of Nuclei Aggregates 	**Description:** In order to obtain single-cell DNA sequencing data, it is important to keep the nuclei aggregates under 5% of the total nuclei. Several factors affect nuclei aggregation including: excessive centrifugation speed, strong pipetting, vortexing, forcing nuclei through cell strainers, tissue/cells over digestion, etc. In addition, if the nuclei suspension concentration is higher than >1.3 x 10^6 nuclei/mL, the overlap of single-nuclei can be observed as false nuclei aggregates (red arrows). **Suggestions:** 1. Do not centrifuge nuclei at high speeds or apply strong mechanic force (mixing, vortexing). 2. If the nuclei concentration is > 1.3 x 10^6 nuclei/mL, dilute the sample and re-quantify. 3. Use only low binding tubes. 4. Verify that the accurate pore size of the strainer's mesh.
Low nuclei recovery	**Description:** The low recovery of nuclei can be caused by different factors. Please review the following recommendations: **Suggestions:** 1. Only use LoBind tubes. The use of other types of plastics can cause the nuclei to attach to the tube walls, reducing the overall yield. 2. Confirm the appropriate initial amount of tissue and the nucleated cellular density of the sample. 3. Verify correct centrifugation speed (500 x g). Lower speeds may reduce overall nuclei yield. Higher speeds may lead to increased nuclei aggregation.
Presence of Cell Debris	**Description:** Solid tumors can often have a higher proportion of dead or dying cells that contribute to high debris. Cell debris can aggregate with nuclei of interest and negatively impact data quality. While counting nuclei, evaluate the level of debris in the sample. Please review the following recommendations for debris removal: **Suggestions:** 1. Debris can be removed by small adjustments to the protocol for centrifugation speeds, wash steps and using the right pore size of strainers mesh. 2. The nuclei can also be separated from cell debris by enrichment methods such as FACS sorting[5] or by density gradient centrifugation using Iodixanol[6]. It is important to have abundant starting nuclei to ensure sufficient end material. 3. Always count nuclei after cell debris removal.

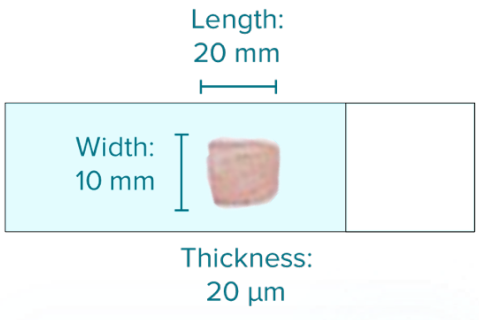
Appendices

Appendix A: Calculation of Tissue Volume Required for Nuclei Extraction

Using a representative H&E slide measure the area of the tissue of interest in mm^2. Multiply the length (mm) and width (mm) of the tissue. If the tissue is not uniform, estimate the area. Next, multiply the area by the tissue section thickness (e.g. 20 µm = 0.02 mm). Finally calculate the total volume by multiplying length x width x thickness (mm^3).

EXAMPLE

A tissue section of 20 µm in thickness is used, 20 mm in length and 10 mm in width. The total tissue volume per section is: 20 mm x 10 mm x 0.02 mm = **4 mm^3**. In this case, two tissue sections are required to achieve the minimum volume of 8 mm^3 for the Nuclei Extraction Protocol. Alternatively, if the thickness of the tissue section is 50 µm, (total volume of 10 mm^3), a single tissue section will yield enough nuclei for processing the nuclei suspension on one Tapestri.



Appendix B: Optimization of Nuclei Extraction

For the enzymatic dissociation protocol, optimizing the lysis incubation time is critical to ensuring that most of the cell membranes are lysed while preventing the over-lysis of the nuclear membrane, which can result in leakage of nuclear contents. We recommend you test different time points to determine optimal lysis incubation time. For sensitive tissue types, a narrower timeline may be appropriate (i.e., unlysed, 1 min, 2 min, 3 min, 4 min.) For robust tissue types, a broader timeline may be appropriate (i.e., unlysed, 2 min, 5 min, 10 min, 15 min). After the completion of each time point, add stop solution and spin down immediately. Remove the supernatant and proceed to assess nuclei quality and yield using fluorescent markers or by using bright field microscopy at higher magnification (60X).–

Nuclei Quality Assessment using Fluorescent Markers (recommended)

Fluorescent markers allow monitoring of both nuclei stability and recovery rate. DAPI is used to stain the nuclear DNA (marker for nuclei). Phalloidin, a heptapeptide with high affinity for the actin filaments (F-actin) of the cell cytoskeleton[7] is used as a cytoplasm marker (GFP, green). Wheat germ agglutinin (WGA), a lectin that specifically binds to N-acetyl-D-glucosamine and N-acetyl-D-neuraminic acid, a component of the cellular membrane glycoconjugates[8] is used, as a membrane marker (RFP, red). Using all three markers allows for assessment of nuclei stability and overall nuclei recovery. In addition, the fluorescent markers serve as a tool to optimize the tissue-specific digestion time.

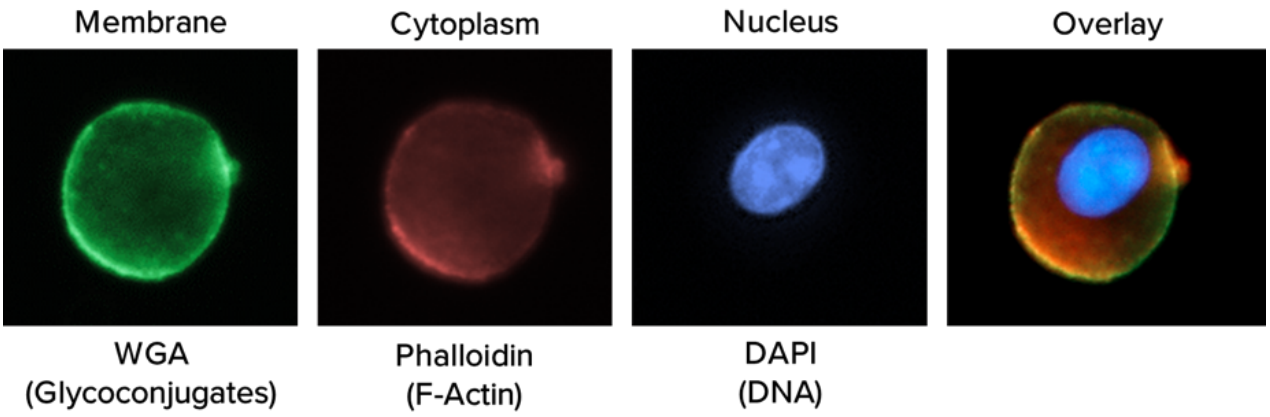
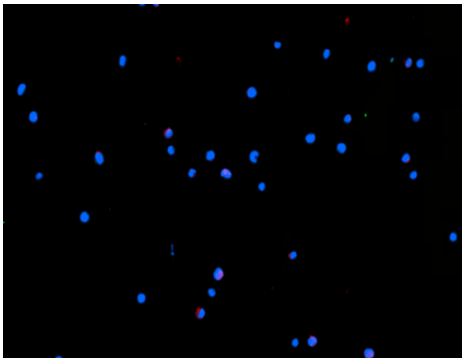
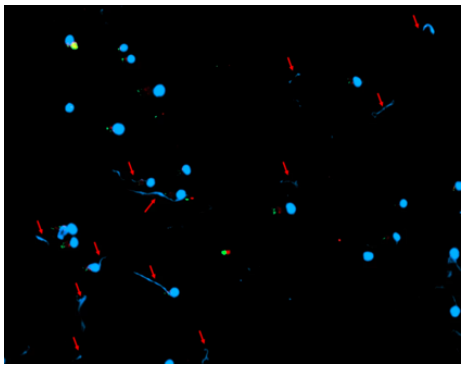
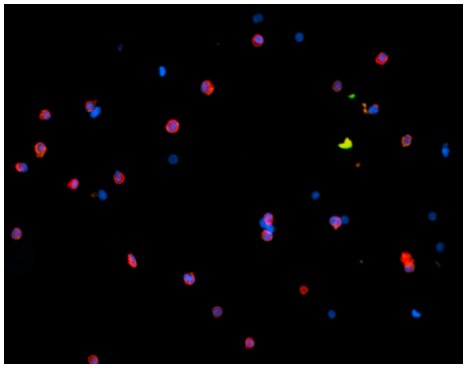
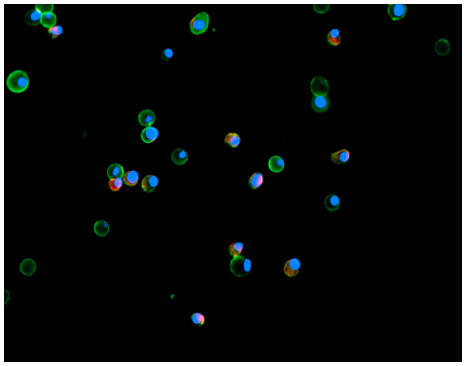


Figure 1. Representative staining patterns of whole cell preparation.

Nuclei Staining Patterns

Optimal nuclei preparation 	The optimal nuclei preparation yields single and intact nuclei (DAPI+). Preserving the nuclear membrane is essential as it provides stability to the nuclei and prevents genomic DNA from leaking. **Recommendation:** If nuclei is intact with no DNA leakage, proceed with confidence.
Over digested nuclei 	DNA (DAPI, blue) leaking outside of the nuclei (red arrows), with very weak or no staining for cytoplasm (RFP, red) or membrane (GFP, green) markers. **Recommendation:** If observed DNA leakage is less than 5 %, proceed with caution; if observed DNA leaking is more than 20 %, do not proceed. If observed leakage is between 5-20 %, proceed at your own risk.
Under digested nuclei  	The nuclei (DAPI, blue) present with strong staining for cytoplasm (RFP, red) or membrane (GFP, green) markers. **Recommendation:** If single cells are observed without clumping, proceed with confidence; if clumping greater than 5% is observed; increase lysis incubation time and re-check under the microscope for clumps (a clump is defined as ≥ 3 aggregated nuclei)

Protocol

A.1 Prepare Phalloidin Solution as follows:

Reagent	Concentration	Volume for Stock 100X
Phalloidin iFluor-555		1 µL
BSA	50 mg/mL	20 µL
PBS	1X	79 µL

IMPORTANT ***Store in the dark at -20°C.***

A.2 Prepare Wheat Germ Agglutinin (WGA) Solution as follows:

Reagent	Concentration	Volume for 1 mg/mL
WGA-Alexa Fluor 488	5 mg	
Mol. Bio. Grad Water		5 mL

IMPORTANT ***Store in the dark at -20°C.***

A.3 **Extract the nuclei** following the steps on pages 13-14 (enzyme dissociation method) or 16-17 (mechanical dissociation) of this document. At the final step the nuclei are resuspended in 100-500 µl of Nuclei Staining Buffer. If the nuclei are already stained with DAPI proceed to step A.5.

A.4 **Add 1 µl of DAPI** (1 mg/mL) to the nuclei suspension.

A.5 **Add 1 µl of WGA Solution** per 60 µl of nuclei.

A.6 **Add 1 µl of Phalloidin Solution** per 60 µl of nuclei.

A.7 **Mix** by gently pipetting up and down.

A.8 Incubate the sample **on ice for 5 minutes.** Protect from light.

A.9 **Dilute the sample 1:3** by mixing 4 µl of the sample with 8 µl of Cell Buffer.

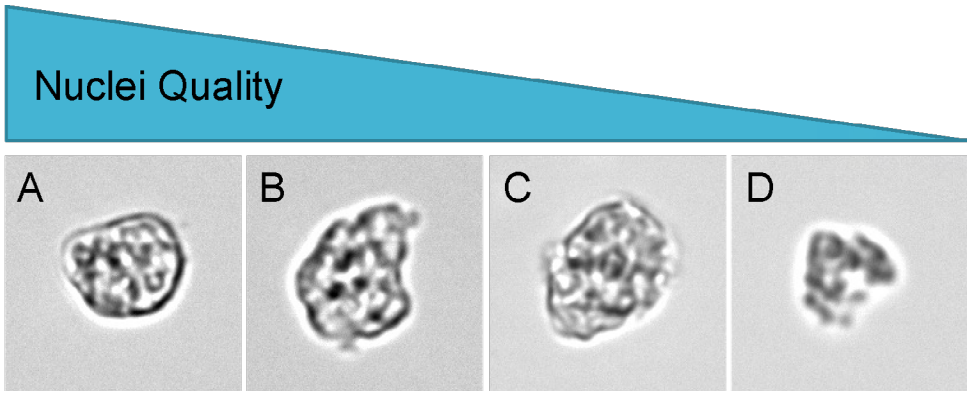
A.10 **Mix** by gently pipetting up and down.

A.11 **Load 10 µl** of the diluted sample on a hemocytometer. Examine the nuclei using the EVOS FL or equivalent fluorescent microscope using the GPF, RFP, and DAPI channels.

A.12 Evaluate the staining patterns of the sample. Assess under- or over-digestion of nuclei. Refer to ***Nuclei Staining Patterns*** on page page 29 of this document for additional information.

Nuclei Quality Assessment by Bright field Microscopy

Higher magnification (60X) is recommended as an additional method to evaluate over digestion and nuclei stability. Dilute an aliquot of the nuclei suspension 1:5 to 1:20 in Cell Buffer. Mix by gently pipetting up and down. Load 10 µl of the diluted sample on a Countess™ Cell Counting Chamber Slide (Thermofisher). Examine the nuclei using the EVOS or equivalent microscope with bright field microscopy at 60X magnification. Evaluate nuclei quality as follows.



A. High-quality nuclei have well-resolved edges. Optimal quality. **Proceed with confidence.**
B. Mostly intact nuclei with minor evidence of blebbing. Little over digested. **Proceed with caution.**
C. Nuclei with strong evidence of blebbing. Medium over digested. **Proceed at your own risk.**
D. Nuclei are no longer intact. Heavily over digested. **Do not proceed.**

References

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