

Takara Bio USA

# Trekker<sup>®</sup> FX Single- Cell Spatial Mapping Kit User Manual

Cat. Nos. SK023, SK024 & SK025

(052026)

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## I. Introduction

This user manual outlines the workflow for using **Trekker® FX Single-Cell Spatial Mapping Kit** (Cat. Nos. SK023, SK024 & SK025) to generate spatially tagged, isolated single nuclei from formalin-fixed paraffin embedded (FFPE) human and mouse tissue samples. These nuclei are subsequently captured using the 10x Genomics [GEM-X Flex Gene Expression Reagent Kits for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture](#) (CG000789, Rev B) to achieve single-cell resolution spatial transcriptomic data from the original tissue section. The Trekker FFPE workflow begins by placing a 10 or 25  $\mu\text{m}$ -thick tissue section onto a standard microscope tissue slide. The Trekker tile slide (referred to as the "Tile" in this document) (Figure 1), a glass substrate embedded with a monolayer of uniquely DNA-barcoded microparticles ("beads"), is then acclimated to room temperature. The tissue slide and tile slide are combined using the Trekker Alignment Fixture, ensuring coverage of the tissue region of interest by the active area of the tile. The assembly is then exposed to UV light, which photocleaves the DNA barcodes and allows them to bind to the tissue. Following spatial labeling, the alignment fixture is disassembled, and the tissue is dissociated into single nuclei. These spatially labeled nuclei are then loaded onto GEM-X Flex Gene Expression Reagent Kits for Multiplexed Samples for single-nucleus (sn) capture and snRNA-seq analysis (Figure 2).

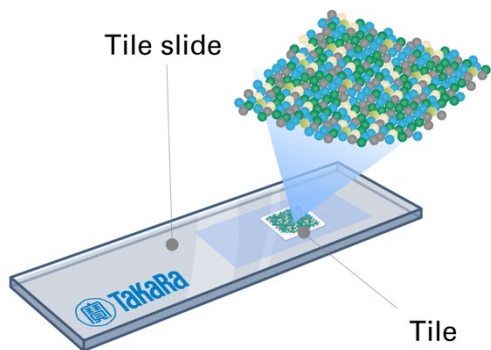


Figure 1. Trekker FX tile.

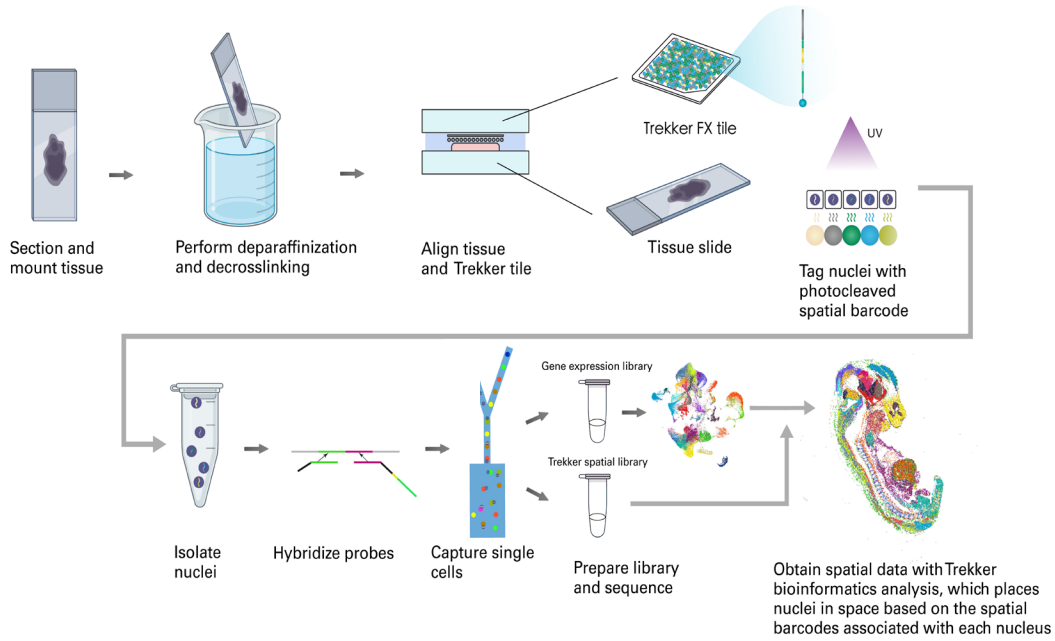


Figure 2. Trekker FX workflow.

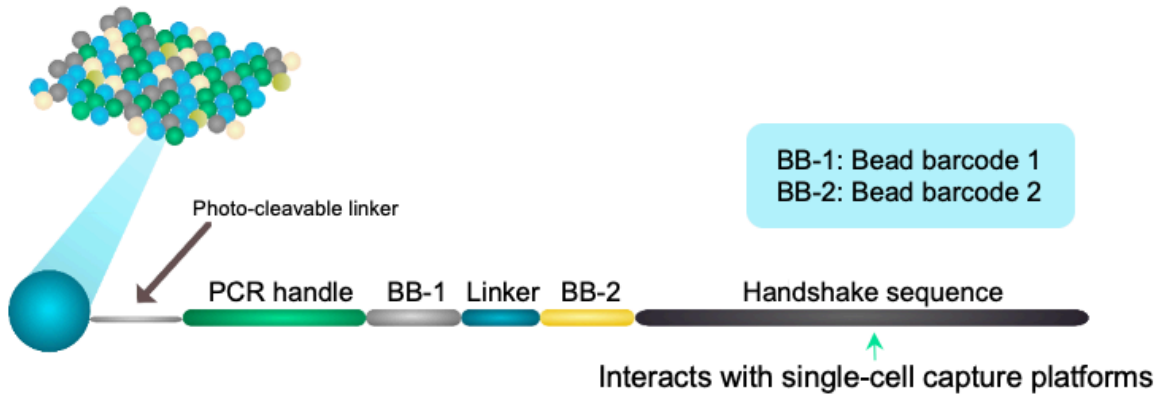


Figure 3. Trekker FX oligo structure.

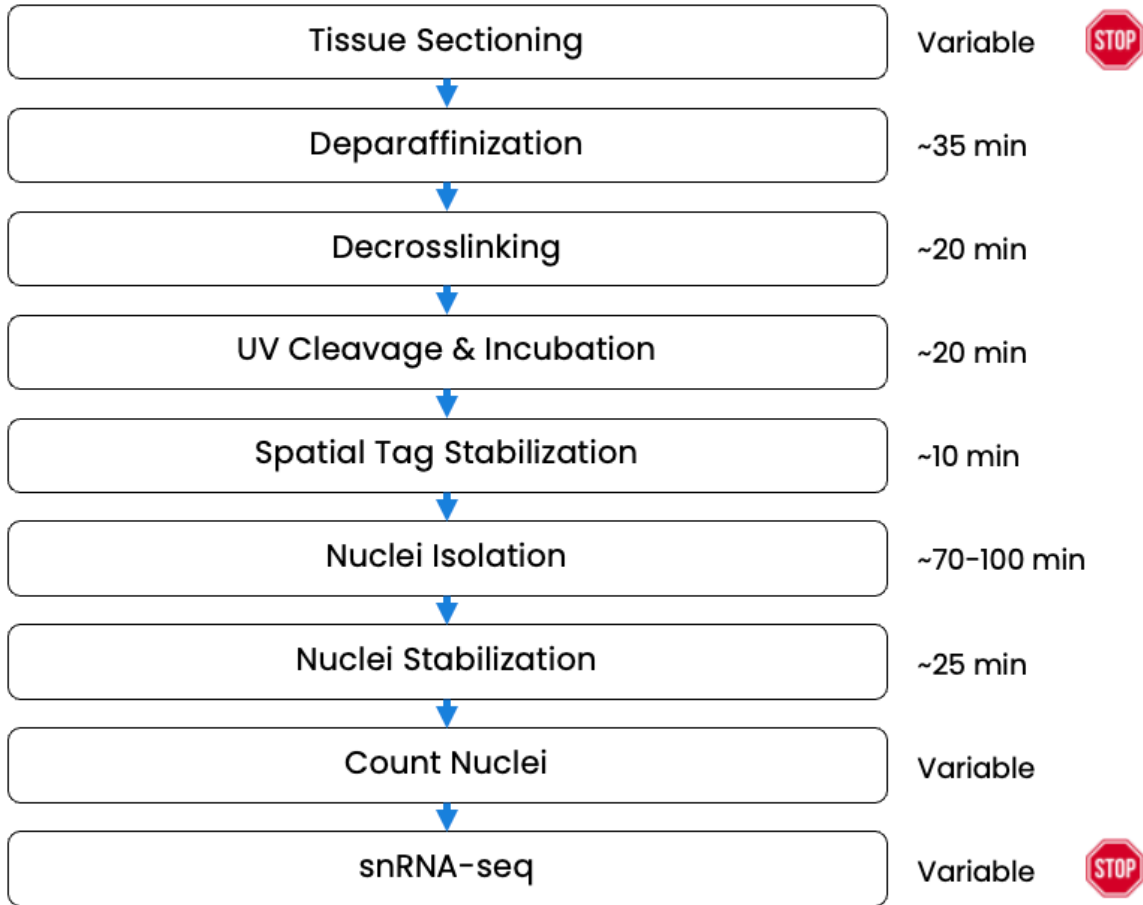


Figure 4. Estimated workflow timing.

## II. List of Components

**Table 1. Trekker FX Single-Cell Spatial Mapping Kit components.** The Trekker FX 10x10 Training Kit Bundle (Cat. No. SK024) is recommended for training or optimization only. It is designed to help optimize the tissue sectioning and nuclei dissociation steps, allowing users to practice and troubleshoot these critical processes.

| <b>Trekker FX 10x10 Bundle</b>   | <b>SK023<br/>(4 Rxns)</b> |
|--|---------------------------|
| <b>Trekker FX 10x10 Tiles (Cat. No. FP012; Store at 4°C)</b>               |                           |
| Trekker FX 10x10 Tiles   | 4                         |
| <b>Trekker FX Reagent Kit (Cat. No. 620001)</b>                            |                           |
| <b>Package 1 (Store at -20°C)</b>  |                           |
| RNase Inhibitor  | 300 µl                    |
| TD Enzyme  | 2                         |
| <b>Package 2 (Store at 4°C)</b>  |                           |
| Nuclei Isolation Buffer  | 4 ml                      |
| Nuclei Wash Buffer   | 25 ml                     |
| BSA-H  | 500 µl                    |
| <b>Package 3 (Store at Room Temperature)</b>                               |                           |
| DX Buffer  | 560 µl                    |
| <b>Trekker FX Accessories (Cat. No. 620002; Store at Room Temperature)</b> |                           |
| Slide-Fixture Adhesives  | 12                        |
| Tile-Slide Spacers A (16–25 µm)  | 2                         |
| Tile-Slide Spacers B (10–15 µm)  | 2                         |
| Slide Mailer   | 4                         |
| <b>Trekker FX 10x10 Training Bundle</b>                                    | <b>SK024<br/>(4 Rxns)</b> |
| <b>Trekker FX 10x10 Training Tiles (Cat. No. TFTB004; Store at 4°C)</b>    |                           |
| Trekker FX 10x10 Training Tiles  | 2                         |
| <b>Trekker FX Training Reagent Kit (Cat. No. 620003)</b>                   |                           |
| <b>Package 1 (Store at -20°C)</b>  |                           |
| TD Enzyme  | 1                         |
| <b>Package 2 (Store at 4°C)</b>  |                           |
| Nuclei Isolation Buffer  | 4 ml                      |
| Nuclei Wash Buffer   | 25 ml                     |
| BSA-H  | 500 µl                    |
| <b>Package 3 (Store at Room Temperature)</b>                               |                           |
| DX Buffer  | 560 µl                    |
| <b>Trekker FX Accessories (Cat. No. 620002; Store at Room Temperature)</b> |                           |
| Slide-Fixture Adhesives  | 12                        |
| Tile-Slide Spacers A (16–25 µm)  | 2                         |
| Tile-Slide Spacers B (10–15 µm)  | 2                         |
| Slide Mailer   | 4                         |

### III. Additional materials required (Not Provided)

**NOTE:** These consumables and reagents have been validated internally by Takara Bio USA, Inc. Substitution is not recommended unless otherwise indicated.

#### **From Takara Bio:**

- Trekker Alignment Fixture Bundle (Cat. No. SK025)
  - Trekker Alignment Fixture (Cat. No. MEC011)
  - Trekker Alignment Fixture Spacers (Cat. No. MEC012)
- Trekker Starter Kit Bundle (UV lamp) (Region-specific, Cat. No. K011, K011EUR, K011UK, or K011AUS)
  - UV Lamp
  - UV Lamp Driver
  - UV Lamp Power Supply (Region-specific)
  - UV Lamp Holder

#### **From 10x Genomics:**

- Chromium X & Accessory Kit (10X Genomics, Cat. No. 1000331)
- 10x Vortex Adapter (10X Genomics, Cat. No. 330002)
- 10x Magnetic Separator HT (10X Genomics, Cat. No. 1000394)
- Chromium X Chip Holder (10X Genomics, Cat. No. 1000393)
- GEM-X Flex Gene Expression Chip Kit, 4 chips (10X Genomics, Cat. No. 1000791)
- GEM-X Flex Sample Preparation v2 Kit (10X Genomics, Cat. No. 1000781)
- Dual Index Kit TS Set A, 96 rxn (10X Genomics, Cat. No. 1000251)
- Dual Index Kit TN Set A, 96 rxn (10X Genomics, Cat. No. 1000250)
  
- 10x Genomics GEM-X Flex v1:
- Fixed RNA Feature Barcode Multiplexing Kit, 64 rxns (10X Genomics, Cat. No. 1000628)
- GEM-X Flex Gene Expression Human 4-plex, 16 samples (10X Genomics, Cat. No. 1000793) or GEM-X Flex Gene Expression Mouse 4-plex, 16 samples (10X Genomics, Cat. No. 1000797) or GEM-X Flex Gene Expression Human 16-plex, 64 samples (10X Genomics, Cat. No. 1000794) or GEM-X Flex Gene Expression Mouse 16-plex, 64 samples (10X Genomics, Cat. No. 1000798)
  
- 10x Genomics GEM-X Flex v2 (Apex):
- GEM-X Flex v2 Human, 4 samples (10X Genomics, Cat. No. 1000926) or GEM-X Flex v2 Mouse, 4 samples (10X Genomics, Cat. No. 1000930) or GEM-X Flex v2 Human, 16 samples (10X Genomics, Cat. No. 1000927) or GEM-X Flex v2 Mouse, 16 samples (10X Genomics, Cat. No. 1000931) or GEM-X Flex v2 Human, 96 samples (10X Genomics, Cat. No. 1000928) or GEM-X Flex v2 Mouse, 96 samples (10X Genomics, Cat. No. 1000932) or GEM-X Flex v2 Human, 384 samples (10X Genomics, Cat. No. 1000929) or GEM-X Flex v2 Mouse, 384 samples (10X Genomics, Cat. No. 1000933)

## From other vendors:

### Reagents & Chemicals

- Ethyl alcohol (Sigma Aldrich, Cat. No. 459844-1L) (or equivalent)
- 10% Neutral Buffered Formalin (Fisher Scientific, Cat. No. 22-050-104) (or equivalent)
- Xylene (Fisher Scientific, Cat. No. 99-905-01) or Histo-Clear (National Diagnostics, Cat. No. 50-899-90147)
- Paraplast (Fisher Scientific, Cat. No. 23-021-752; 50-276-93) (for paraffin embedding)
- RNase, DNase free water (Thermo Scientific, Cat. No. J71786-K8) (or equivalent)
- Hematoxylin & Eosin Staining Kit (Abcam, Cat. No. ab245880) (or equivalent)
- 0.1 N HCl (RICCA, Cat. No. 3600-4) (or equivalent)
- 32% Paraformaldehyde (Electron Microscopy Sciences, Cat. No. 15714) (or equivalent)
- Phosphate-Buffered Saline, 1X without calcium and magnesium, PH 7.4 ± 0.1 (Corning, Cat. No. 21-040-CV) (or equivalent)
- Tris Buffer (1M, pH 8.0) (VWR, Cat. No. E199-100ML) (or equivalent)
- AO/PI or Ethidium Homodimer-1 (highly recommended)
- DAPI (Thermo Fisher Scientific, Cat. No. 62248)
- Silicone lubricant (CRC, Cat. No. 05074) (for long-term maintenance of the fixture only)

### Histology & Staining Supplies

- Superfrost Plus Microscope slides (Fisher Scientific, Cat. No. 1255015)
- Staining Boxes (Thermo Scientific, Cat. No. 5705-1010; Bel-Art, Cat. No. 03-410-491) (or equivalent)
- Glass Coplin staining jars (Epredia, Cat. No. E94) (or equivalent)
- Slide drying rack (Medicus Health, Cat. No. NC1510992, NC1510993) (or equivalent)
- Automated slide stainer (Rankin, Cat. No. STN65) (or equivalent)
- Microtome (or equivalent)
- Microtome blades (or equivalent)
- Sectioning brushes (or equivalent)
- Tissue floatation bath (room temperature to 70°C minimum range) (Newcomer Supply, Cat. No. 6950) (or equivalent)
- Single-edge razor blades (Ihc World, Cat. No. IW-2100) (or equivalent)
- Slide mailer (Fisher Scientific, Cat. No. HS15986) (or equivalent)
- Low-lint Kimwipes (Kimberly-Clark, Cat. No. S47299) (or equivalent)

### Labware & Consumables

- pluriStrainer Mini 20 µm (Cell Strainer) or pluriStrainer Mini 40 µm (Cell Strainer) (pluriSelect, Cat. Nos. 43-10020-50 or 43-10040-50, respectively)
- Filter pipette tips: 20 µl, 200 µl, and 1,000 µl (Rainin, Cat. Nos. 30389226, 30389240 & 30389213) (or equivalent)
- Single-channel pipette: 10 µl, 20 µl, 200 µl, and 1,000 µl (Rainin, Cat. Nos. 17014388, 17014392, 17014391 & 17014382) (or equivalent)
- DNA LoBind 1.5 mL tubes (Eppendorf, Cat. No. 022431021) (or equivalent)
- DNA LoBind 5 mL tubes (Eppendorf, Cat. No. 0030108310) (or equivalent)

- Protein LoBind 1.5 mL tubes (Eppendorf, Cat. No. 022431081) (or equivalent)
- 15 mL centrifuge tubes (Falcon, Cat. No. 352095) (or equivalent)
- 50 mL centrifuge tubes (Falcon, Cat. No. 352070) (or equivalent)
- Tweezers (Ted Pella, 58083-NM) (or equivalent)

## Equipment & Instruments

- TapeStation (Agilent, Cat. No. G2992AA)
- Ice bucket (Cole-Parmer, Cat. No. EW-04393-66) (or equivalent)
- FFPE cooling tray (Histo Cool Block, Cat. No. NC0749593) (or equivalent)
- Oven (room temperature to 100°C minimum range) (or equivalent)
- Desiccator Cabinet (SP Bel-Art, Cat. No. F42071-0000, F42072-1000) (or equivalent)
- Small desk/table fan (JisuLife, Cat. No. FA28) (or equivalent)
- Water or Bead bath (25°C to 100°C minimum range) (Thermo Scientific, Cat. No. TSGP02, TSGP2S) (or equivalent)
- Thermomixer (Eppendorf, Cat. No. 5382000023) (or equivalent)
- Thermal cycler (Bio-Rad, Cat. No. 12015392) (or equivalent)
- Bioanalyzer (Agilent, Cat. No. G2939B) (or equivalent)
- Fluorescence microscope (or equivalent)
- Automated fluorescent cell counter (or equivalent)
- Refrigerated centrifuge with swing bucket rotor (or equivalent)
- Mini centrifuge for 1.5 ml tubes (or equivalent)
- Mini centrifuge for 0.2 ml tubes (or equivalent)
- Vortexer (or equivalent)

## IV. General Considerations or Precautions

### A. Tissue requirements and recommendations for assessing quality

- The Trekker FX assay is developed and optimized for formalin-fixed paraffin-embedded tissues. Application of other sample formats is not supported. Contact technical support for supported applications ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)).
- Assess the RNA quality of your tissue by collecting four 10 µm sections and isolating RNA with the RNeasy FFPE Kit (Qiagen Cat. No. 73504) or equivalent. Alternatively, sections can be collected on slides, deparaffinized, scraped with a razor blade, and then collected for RNA extraction. Analyze the extracted RNA from your sections on an Agilent Bioanalyzer or TapeStation to derive a DV200 score (the percentage of RNA fragments >200 nucleotides). Good quality RNA should have a DV200 score >40%. Spatial positioning rates may be lower for tissue samples with low DV200 scores. **It is not recommended to perform Trekker FX on samples with a DV200 score of <40%.**
- Performance could be impacted by tissue composition, such as the presence of necrotic regions, dense/fibrotic tissues, and/or endogenous RNase activity within the sample.
- Assess tissue quality and morphology by performing H&E staining on a section adjacent to the one used for the Trekker FX workflow.
- Verify that the tissue fits within a 10 x 10 mm area. If too large, identify the region of interest and trim the section according to Step 1.4 of the Trekker FX workflow.
- Depending on the desired nuclei yield, practice and optimize 10 or 25 µm thick section generation and collection. Thicker sections will yield more intact nuclei than thinner sections. For example, 25 µm mouse

brain sections can yield 6-fold more nuclei than 10 µm sections. Note that 16–25 µm thick sections would require Tile-slide Spacer A, while 10–15 µm thick sections require Tile-slide Spacer B for spatial tagging alignment.

- Be aware that approximately 40–50% nuclei loss is expected from overnight hybridization, and post-hybridization washes in the 10x Chromium Flex workflow. Thus, it is critical to identify the appropriate thickness and starting nuclei yield prior to the start of the Trekker FX assay.
- Be aware that different tissue types and upstream tissue handling protocols may impact the final nuclei yields. When possible, optimize and determine the ideal nuclei yields with the Trekker FX 10x10 Training Kit (SK024) prior to executing the Trekker FX 10x10 assay kit (SK023).
- Tissue optimization and nuclei isolation tests can be performed without the tile using the buffers provided in the Trekker FX 10x10 Training Kit.

## B. Tips and Techniques

- Ensure that working surfaces are clean and free of nucleases and debris.
- Ensure that assay reagents are prepared fresh and stored on ice, unless otherwise instructed in the protocol. Deparaffinization reagents can be prepared in advance.
- Ensure adhesive Superfrost Plus Microscope Slides are used for tissue collection and mounting.
- Ensure that deparaffinization is complete and the section is free of paraffin.
- Wear protective UV glasses when working with the UV lamp and **DO NOT** stare directly into the lamp during operation.
- The beads on the Trekker tiles contain photocleavable oligos. Store the tiles appropriately to avoid light exposure until ready to use.
- Perform the whole protocol on ice as much as possible, unless otherwise instructed in the protocol.
- Keep buffers, tissues, and nuclei suspension cold throughout the workflow, unless otherwise instructed in the protocol.
- Always wear gloves when handling the tiles and avoid direct contact with the beads prior to UV cleavage.
- Verify that the tissue region of interest fits within a 10 x 10 mm area, otherwise trim according to Step 1.4 of the Trekker FX workflow.
- Some tissue types may have larger and more sparse nuclei. To increase the number of nuclei recovered, tissue section thickness may be increased (up to 25 µm) and/or using a cell strainer with a larger pore size (40 µm, see “Labware & Consumables” in Section III).
- When generating thicker FFPE sections, ensure the microtome is properly calibrated and that the tissue block is thoroughly hydrated in nuclease-free water. For more details, see [Tissue and Section Preparation](#) and [Troubleshooting](#).
- For filtering nuclei with the pluriStrainer Mini cell strainer, hold the pipette tip at an angle and gently touch the filter membrane where it meets the filter wall. Slowly pipette the liquid through the filter. If necessary, reposition the pipette tip on the membrane to allow for continuous flow.
- If any liquid remains in or at the base of the filter, gently pipette any remaining volume from underneath the filter and transfer to the collection tube.
- For all centrifugation steps, use a swing bucket centrifuge. This will enable the formation of a tight pellet at the base of the tube and minimize loss during supernatant aspiration. Avoid using a fixed angle centrifuge as this would lead to reduced yields and recovery.

### C. Optimizing Nuclei Isolation

- It is recommended to evaluate nuclei isolation quality for your tissue type before performing the Trekker FX experiment. Some tissue types may require further optimization from the standard protocol and reagents provided.
- A successful dissociation should result in good nuclei yield (>50% of the number of expected cells in the section for your tissue type) and purity (>75% single intact nuclei without excessive clumping and debris) (Figure 5).
- To accurately count and assess the quality of nuclei, we recommend using a fluorescence microscope with AO/PI or another fluorescent marker to distinguish nuclei from debris. It is recommended to check nuclei under higher magnification (20x–40x) to evaluate nuclei quality. High quality nuclei should maintain membrane integrity. Excess blebbing indicates over-lysis.
- If yield is low, check the final suspension for signs of under-lysis (undigested pieces of tissue) or over-lysis (nuclei blebbing or lysing). You may need to increase or decrease the dissociation time in Step E.8. If the quality of the nuclei is poor (over-lysis), reducing the dissociation time and trituration rounds may improve the nuclei quality.
- If necessary, assess the nuclei quality at different timepoints during dissociation to ensure the presence of brightly stained nuclei and the absence of nuclei blebbing.
- Count nuclei with AO/PI or Ethidium Homodimer-1 and dilute the nuclei to the desired concentration based on the guidelines for your single-cell platform of choice. Use of Trypan Blue alone can lead to overestimated nuclei counts, as it is difficult to differentiate between nuclei and debris.
- Concentrations that are too high or too low concentrations may lead to inaccurate results during counting.
- Contact technical support for additional guidance ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)).

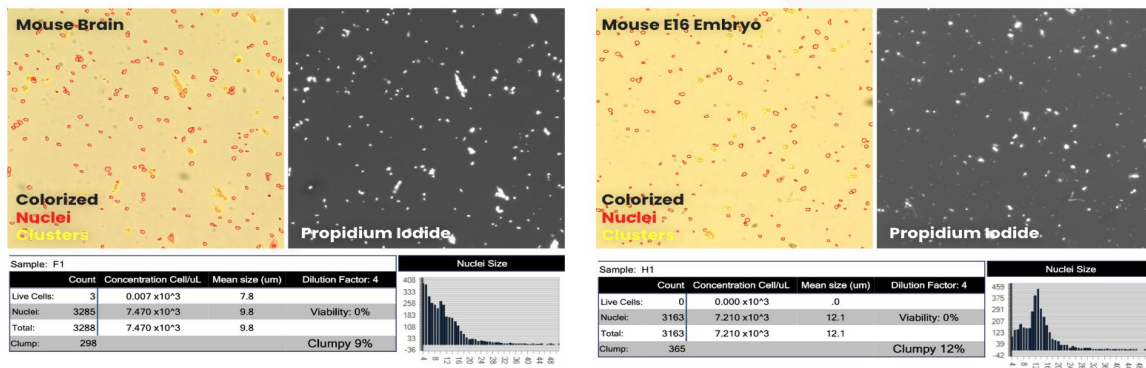


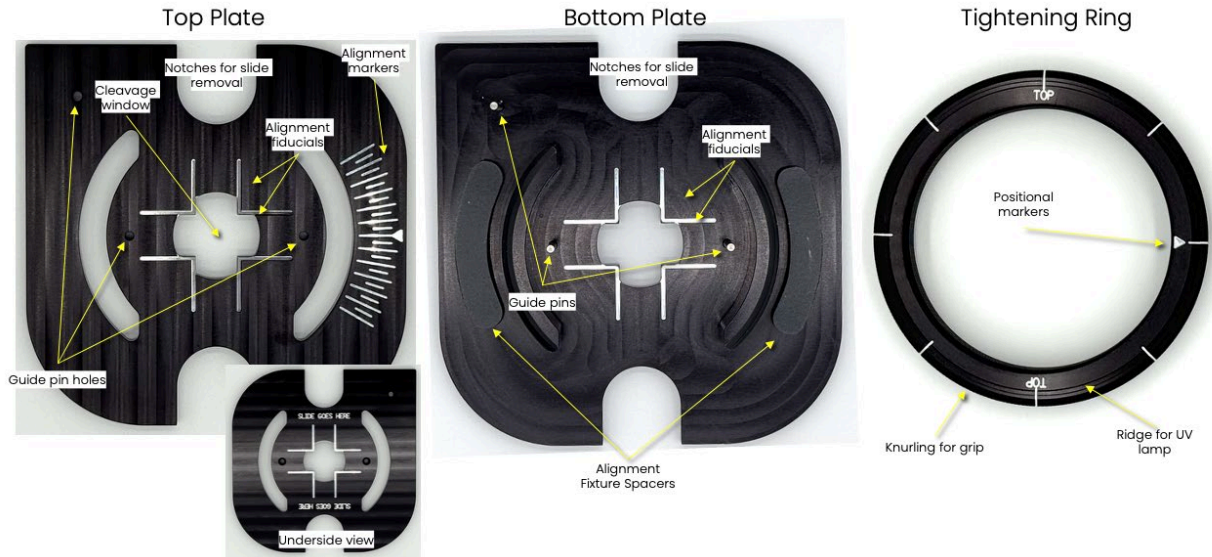
Figure 5. Representative isolated nuclei from mouse brain and E16 embryo. Output images and metrics taken from the Revvity Ascend. Nuclei were isolated from a single 25 µm thick mouse brain coronal section and whole E16 embryo.

## V. Alignment Fixture Assembly and Disassembly

This section provides detailed instructions for how to assemble and disassemble the Trekker Alignment Fixture. It is recommended for users to review this section carefully before performing next steps.

### A. Assembly

1. The Trekker Alignment Fixture is made up of three components (Figure 6):



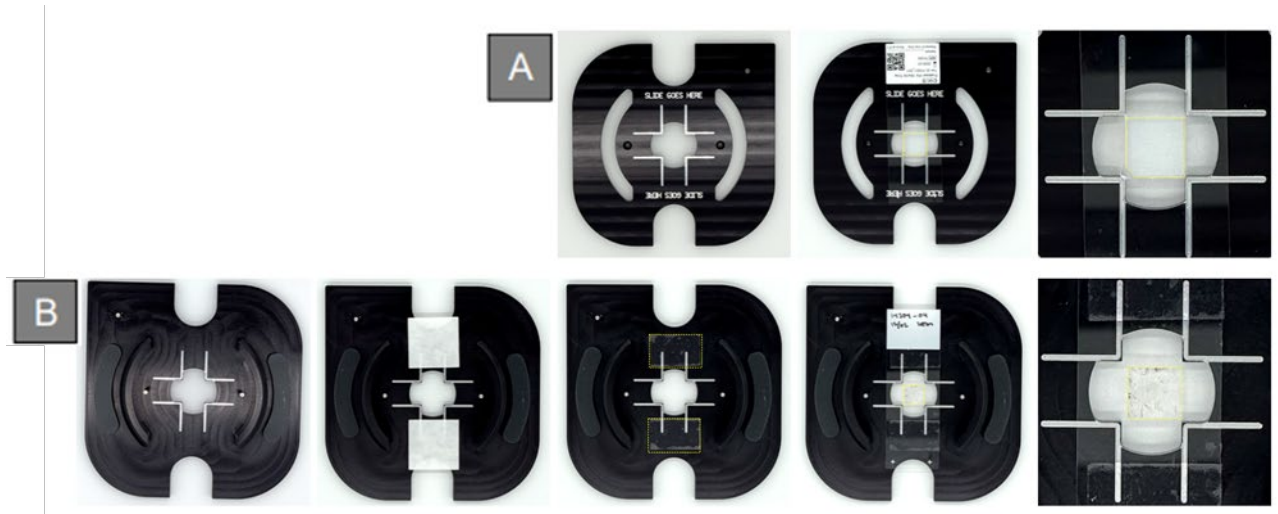
**Figure 6. Alignment Fixture components:** Top Plate, Bottom Plate, and Tightening Ring. The Top Plate is designed to hold and align the Trekker FX Tile, while the Bottom Plate is designed to hold and align the Tissue slide. The Tightening Ring is used to allow for the controlled sandwiching of the Trekker FX Tile and Tissue slide.

2. The Trekker FX 10x10 Tile and the Tissue slide are designed to fit within the Top Plate and Bottom Plate of the Trekker Alignment Fixture, respectively.
3. During fixture assembly, note positioning of the asymmetric corner and ensure the sides of the tile align to the fiducials of the top plate.
4. On the bottom surface of the Top Plate, place the provided double-sided slide-fixture adhesive on the areas indicated in the boxes (Figure 7a). Make sure that the central cleavage window is not obstructed.



**IMPORTANT:** Verify that there are no folds or creases in the double-sided adhesive, as these would impact the distance between the Tissue slide and tile, and affect the efficiency of spatial tagging.

5. Install the Trekker FX 10x10 Tile on the Top Plate by removing the backing of the adhesive and aligning the active area to the fiducials and making sure it is centered in the cleavage window (Figure 7a).
6. Press firmly to ensure the slide is adhered to the Top Plate and fully flush.
7. On the top surface of the Bottom Plate, place the provided double-sided tape on the areas indicated in the boxes (Figure 7b). Make sure that the cleavage window is not obstructed.
8. Install the Tissue slide onto Bottom Plate, making sure it is centered in the window and aligns with the fiducials (Figure 7b). If necessary, rotate the Tissue slide to maximize the tissue-tile coverage.
9. Press firmly to ensure the slide is adhered and flush.

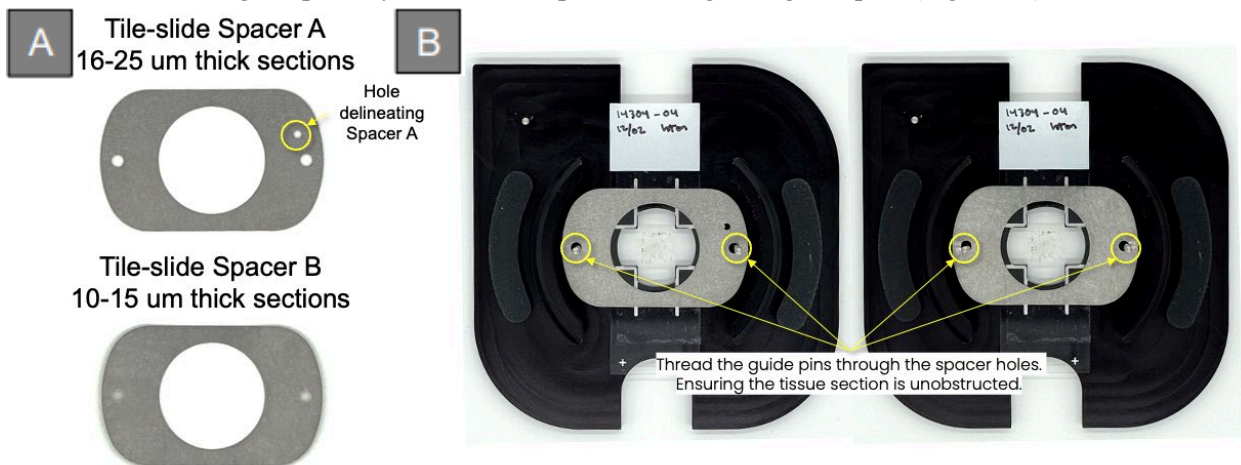


**Figure 7. Step-by-step attachment of the Trekker FX Tile and Tissue slide on the Top Plate (Panel A) and Bottom Plate (Panel B), respectively.** The active area of the Trekker Tile and the tissue must be within the boxed area formed by the fiducials (dotted yellow line).



**IMPORTANT:** Failure to properly align either the Trekker FX Tile or Tissue slide to the fiducials may lead to inaccurate or inefficient spatial tagging of the tissue.

10. After attaching the Tissue slide to the Bottom Plate of the Trekker Alignment Fixture, install either Tile-slide Spacer A for tissue sections 16–25  $\mu\text{m}$  thick or Tile-slide Spacer B for tissue sections 10–15  $\mu\text{m}$  thick (Figure 8a).
11. Install the Tile-slide Spacer by aligning the cleavage window of the spacer to the cleavage window of the fixture and threading the primary holes of the spacers through the guide pins (Figure 8b).



**Figure 8. Positioning of the Tile-slide spacers.** **Panel A.** Side-by-side view of the Tile-slide Spacer A (top) and B (bottom), with Spacer A delineated with a third hole. **Panel B.** Diagram depicting the installed Tile-slide Spacer A (left) and Spacer B (right) on the Trekker Alignment Fixture Bottom plate. Ensure that the primary holes are aligned with the guide pins and the tissue section is not obstructed.

12. After application of the Cleavage Buffer, proceed to combine the Top Plate and Bottom Plate so that the Trekker FX Tile and Tissue slide are facing each other (Figure 9).
13. Ensure that the asymmetric corners of the top and bottom plates and the respective guide pins align, and the Trekker Tile active area is overlapping with (but not touching) the tissue.

14. Install the Tightening Ring at the top of the thread, with the arrow pointing toward the guide pin at the top-left corner of the assembly (circle in Figure 9). If necessary, slowly rotate the ring clockwise and counterclockwise to ensure the threads are aligned and engaged.
15. Slowly rotate the Tightening Ring clockwise to merge the top and bottom plates and the corresponding slides. Ensure that the threads are aligned and **DO NOT** forcibly tighten the Tightening Ring.

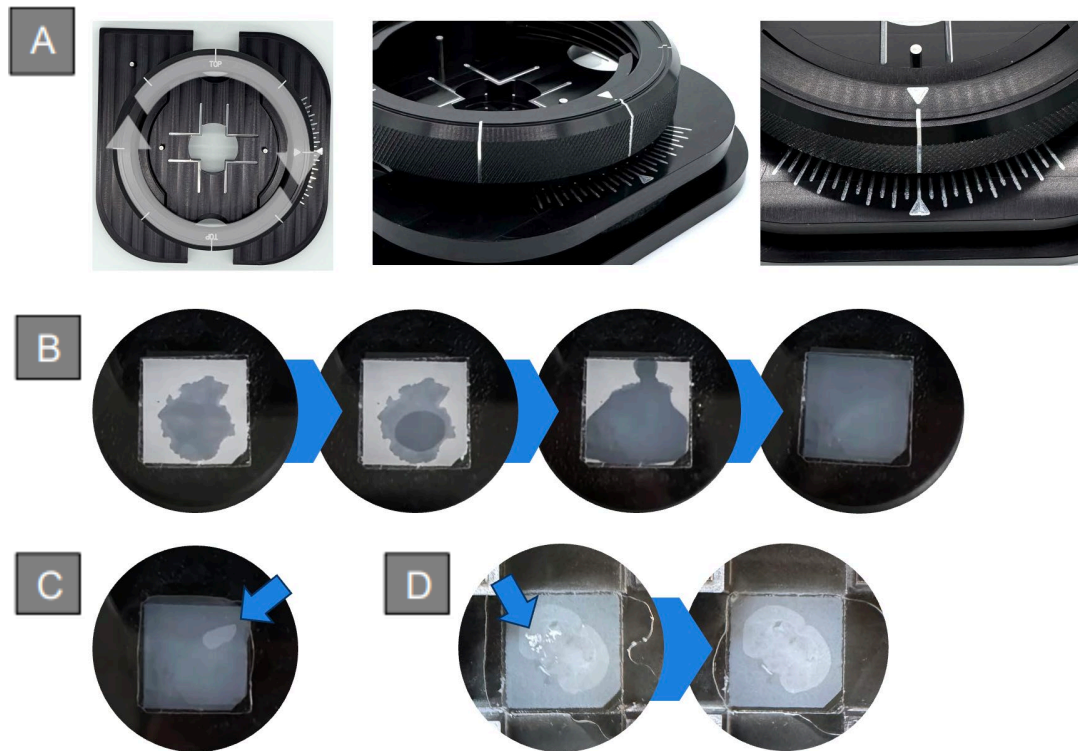


**IMPORTANT:** Ensure that the threads of the Bottom Plate and Tightening Ring are aligned and engaged. If the assembly is not smooth, then the threads are not aligned (i.e., cross-threaded). If this occurs, **DO NOT** forcibly tighten the fixture. Instead, spray silicone lubricant directly on the threads, allow to soak for >5 min and carefully disengage the ring and plate by rotating counterclockwise. Using excessive force may damage the Trekker Alignment Fixture and impact its performance.



**Figure 9. Step-by-step assembly of the Trekker Alignment Fixture.** Align the asymmetric corners of the Top and Bottom plates, then combine the plates with the Tile and Tissue slide facing each other. Install the Tightening ring, with the arrow pointing toward the top-left guide pin to engage the threads and turn clockwise to sandwich the two slides.

16. Continue to tighten the ring until the arrow markers on the Tightening Ring and Top Plate align (Figure 10a) and a visible meniscus forms and covers the tile (Figure 10b). If an air bubble is present (Figure 10c), slowly and gently loosen the ring and repeat tightening until the bubble is absent. If the tissue begins to touch the tile surface (Figure 10d), slowly and gently loosen the ring until the tissue appears uniform.



**Figure 10. Controlled merging of the Trekker FX 10x10 Tile and tissue within the Alignment Fixture.** **Panel A.** Slowly turn the tightening ring clockwise until the arrow markers align. **Panel B.** Simultaneously, observe the Cleavage Buffer meniscus covering both the tile and tissue. **Panel C.** If a bubble is trapped between the slides (arrow), loosen and retighten the ring until the bubble is absent. **Panel D.** If the tissue begins touching the tile (arrow), loosen the ring until the tissue appears uniform.

17. At this point, the Trekker Alignment Fixture is fully assembled. Proceed with UV cleavage and incubation.



**IMPORTANT:** Use of the thinner Tile-slide Spacer B will result in the Tightening Ring going past the arrow marker of the Top plate. Ensure that the Cleavage Solution thoroughly covers the tissue and the tile.



**IMPORTANT:** If bubbles are present between the tissue and Tile, **DO NOT** proceed with cleavage as this may lead to improper or inefficient spatial tagging. Disassemble and reassemble until no bubbles are present.



**IMPORTANT:** Tissue sections may expand during processing and may touch the tile during assembly. If the tissue touches the tile, **DO NOT** proceed with cleavage. Gently and slowly loosen the ring until the tissue appears uniform.

## B. Disassembly

1. To disassemble the Trekker Alignment Fixture, slowly rotate the Tightening Ring counterclockwise to separate the Top and Bottom plates (Figure 11).
2. Gently remove the Tightening Ring and Top Plate without touching the Trekker FX Tile and tissue.



**Figure 11. Step-by-step disassembly of the Trekker Alignment Fixture.** Gently rotate the Rightening Ring counterclockwise to slowly separate the Top and Bottom plates. Carefully remove the Tightening Ring and the Top Plate without touching the tissue. Remove the Tissue slide from the Bottom Plate and proceed to the next steps of the protocol.

3. Carefully remove the Tissue slide from the Bottom Plate and place on a flat surface.
4. Proceed with Trekker FX washes, filtration, isolation, and stabilization.
5. After use, carefully remove the Trekker FX Tile and any remaining adhesives.
6. Discard the slides in an appropriate glass or sharps waste container.
7. After each use, proceed to Maintenance.



**IMPORTANT:** If the Top and Bottom Plates fail to separate during disassembly or the top and bottom slides touch prematurely during assembly, the foam Trekker Alignment Fixture Spacers may need to be replaced. Remove the existing foam spacers, clean the surface with ethanol, and then allow it to dry. Remove the backing of replacement foam spacers to expose the adhesive surface and then install 5 mm to the side of the bottom plate threads.

## C. Maintenance

1. After or prior to each use, disassemble the Trekker Alignment Fixture and ensure that the surfaces are free of adhesive or residue.
2. Lightly spray all Trekker Alignment Fixture surfaces with 70% ethanol.
3. Using a lint-free Kimwipe, wipe all surfaces until dry.
4. For deep cleaning, spray a Kimwipe with 70% ethanol and thoroughly wipe the threads of the Tightening Ring and the Bottom Plate.
5. Allow to completely dry for 15–30 min at room temperature.
6. Store the Trekker Alignment Fixture disassembled in a dry environment at room temperature.

**TIP:** If one or both foam Trekker Alignment Fixture Spacers are contaminated or show signs of deterioration, proceed to remove during Step 3.1. Then apply a new foam spacer after Step 3.10 prior to storage.

**TIP:** If the assembly of the tightening ring is met with friction or chattering, then cleaning and additional silicone lubricant between the threads is necessary. Clean and wipe dry the Trekker Alignment Fixture with 70% ethanol. Then lightly spray with silicone lubricant and coat the threads of the Tightening Ring and Bottom Plate. Then, assemble the Bottom plate and Tightening Ring and proceed to rotate the components at least 5x until the thread surfaces are thoroughly coated. Wipe off any excess residue and repeat process until it can be smoothly operated. Wipe off any excess residue and store at room temperature.

## VI. Tissue and Section Preparation

The following section provides guidance on tissue and sample preparation. Depending on the tissue size, type, and composition it may be necessary to modify the Tissue Processing, Sectioning, and H&E staining protocol. It is important for the user to perform the appropriate assessment and optimizations prior to the start of the Trekker FX assay.

### A. Tissue processing

The following FFPE tissue processing protocol provided is intended solely as an illustrative example. FFPE processing workflows, reagents, fixation times, equipment, and quality control standards may vary significantly between institutions, laboratories, and processing centers based on local practices, regulatory requirements, tissue type, and downstream applications. It is the sole responsibility of the user to verify, validate, and optimize the protocol for their specific site and use case. Users must ensure that tissues are adequately fixed, consistently processed, and uniformly infiltrated with paraffin wax to achieve acceptable morphological preservation and molecular performance. Prior to downstream analytical use, users should perform appropriate quality control, validation, and documentation to confirm that FFPE tissue blocks meet the required standards for their intended applications. For additional information, refer to the [Troubleshooting](#) section for possible root causes and solutions.

1. After dissection or resection, place fresh tissue in 10% Neutral Buffered Formalin (NBF) at >10x the volume of the tissue.
2. Fix for 6–48 hr at room temperature, or 24–48 hr at 4°C.
3. Proceed with tissue dehydration, clearing, and wax infiltration. Example protocol presented below:

| Step | Reagent  | Time (min) | Temperature (°C) |
|------|--|------------|------------------|
| 1    | 70% ethanol  | 44         | 45               |
| 2    | 100% ethanol   | 30         | 45               |
| 3    | 100% ethanol   | 30         | 45               |
| 4    | 100% ethanol   | 30         | 45               |
| 5    | 100% ethanol   | 30         | 45               |
| 6    | 100% ethanol   | 60         | 45               |
| 7    | 100% ethanol<br><i>Note: Steps 2-7 consist of an ascending EtOH gradient beginning with a concentration of 80% and ending with a concentration of no less than 98%.*</i> | 90         | 45               |
| 8    | Xylene   | 45         | 45               |
| 9    | Xylene   | 45         |                  |
| 10   | Xylene<br><i>Note: Steps 8-10 consist of an ascending xylene gradient ending with a concentration of no less than 95%.*</i>  | 90         | 45               |
| 11   | Paraplast paraffin   | 60         | 65               |
| 12   | Paraplast paraffin   | 60         | 65               |

|    |                    |    |    |
|----|--------------------|----|----|
| 13 | Paraplast paraffin | 80 | 65 |
|----|--------------------|----|----|

*Note: Steps 11-13 consist of an ascending paraffin gradient ending with a concentration of no less than 95%.\**

\*Concentrations are determined by the on-board hydrometer.

Choose an appropriately sized mold and cassette that will accommodate the tissue area and depth.

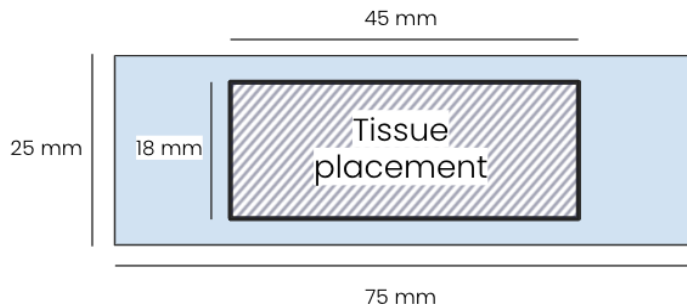


**IMPORTANT:** Improperly sized paraffin molds or cassettes relative to the tissue size may lead to difficulty in sectioning. Use a mold or cassette that allows for the paraffin wax to surround and cover the tissue.

4. Fill the mold with molten paraffin wax and place the tissue in the correct orientation.
5. Place the labeled cassette on top of the molten paraffin.
6. Transfer to a cold plate and allow the paraffin to solidify.
7. Once solid, store the FFPE block in a sealed container, preferably in a 4°C fridge to minimize analyte degradation.

## B. Sectioning

1. Place the labeled cassette on top of the solidified paraffin.
2. Clean all surfaces with 70% ethanol and wipe dry with a Kimwipe before and after each sectioning session.
3. Prepare a warm water bath with nuclease-free water and set it to 40–42°C. Ensure there are no bubbles on the base or surface of the water bath.
4. Prepare a cold bath with wet ice made with nuclease-free water. Nuclease-free ice can be made ahead of time by freezing nuclease-free water.
5. Prepare tissue collection slides by marking the recommended collection area as depicted in Figure 12, below.



**Figure 12. Tissue placement for tissue collection slides.** Using a full-page printout of this page, place the tissue collection slide on the figure above. On the backside of the slide, mark the borders of the tissue placement area to serve as guides for tissue placement. The area corresponds to the accessible regions of the Trekker Alignment Fixture.

6. Incubate the FFPE tissue block on nuclease-free wet ice for 15–30 min.
7. Prior to installation, spray the microtome blade with 70% ethanol and carefully wipe dry with a lint-free Kimwipe to remove any protective oil or debris from the surface.
8. Prior to installation, blot the tissue block dry with a lint-free Kimwipe.
9. Install the FFPE block on the microtome, verifying the positioning and orientation.

10. Face the FFPE tissue block by sectioning and trimming until the desired depth and region of interest is reached.

**TIP:** Use this opportunity to adjust the block positioning, alignment, and thickness on the microtome to ensure consistency of tissue section generation. Depending on the tissue size and block, excess paraffin from the block edges can be carefully removed with a single-edge razor blade. However, retain a sufficient border of wax to maintain the section structure during sectioning and handling.

**TIP:** Confirm the desired region of interest (ROI) by collecting 5–10  $\mu\text{m}$  sections. Then perform a quick deparaffinization, H&E staining, and cover slipping, followed by imaging on a microscope.



**IMPORTANT:** Use adhesive Superfrost Plus Microscope Slides for tissue section placement and collection. DO NOT use non-adhesive microscope slides as this will lead to potential detachment at later steps and reduced nuclei yields.

11. Once the desired region has been reached, proceed with section collection.
12. Set the microtome to the desired thickness (10 to 25  $\mu\text{m}$ ).



**IMPORTANT:** Selection of thickness is dependent on the desired nuclei yield and output. Thicker sections will generate higher nuclei yield following isolation and single-cell prep, but may impact H&E staining/imaging quality. Thinner sections will generate higher quality H&E staining/images, but may impact the nuclei yield and spatial coverage.

13. Slowly section the block while simultaneously pulling down with the brush to keep the section flat and minimize detachment from the surrounding paraffin.
14. Using the brush or forceps, carefully transfer the section to the pre-warmed water bath while avoiding any debris or bubbles on the surface.



**IMPORTANT:** If visible cracking or chattering of the tissue is observed, immediately **STOP** sectioning. The tissue section might be overly dehydrated. Remove the tissue block and rehydrate by incubating on wet ice for >15 min. Repeat Steps B.6 to B.11. If the problem persists, refer to the [Troubleshooting](#) section for possible root causes and solutions.

15. Incubate the section in the warm water bath for 6–8 min (depending on tissue type. See Table 2) to flatten the section and remove wrinkles.
16. Once flattened, float the section to the tissue placement area of the collection slide, and gently remove the slide with the tissue from the water bath.
17. Remove any excess water from the slide using a Kimwipe to wipe the surface without touching the tissue.
18. Place the Tissue slides vertically on a slide drying rack with the desk fan blowing air towards the slides.
19. Allow the Tissue slides to dry at room temperature for 15–20 min (for most tissues) until water is visibly absent from underneath the section.



**IMPORTANT:** The amount of time needed for the tissue section to flatten and dry will depend on the tissue type and condition of the block. Allowing too little or too much flattening time may result in wrinkled sections or breaking up of the tissue, respectively. Allowing too little or too much drying time may result in water being trapped underneath the section or the section detaching from the slide (see example times Table 2).



**IMPORTANT:** If sections are being collected from different tissue blocks, tissue types, or other species, use proper contamination control to minimize cross contamination. Between blocks you should ideally clean the instrument and the area with 70% ethanol and wipe dry with a Kimwipe, and replace the sectioning blade.

| Species | Tissue type                    | Status   | Section thickness (µm) | Warm water bath incubation (min) | Room temperature drying time (min) |
|---------|--------------------------------|--|------------------------|----------------------------------|------------------------------------|
| Mouse   | Brain (olfactory bulb)         | Healthy  | 25                     | 6–8                              | 15–20                              |
|         | Brain (forebrain)              | Healthy  | 25                     | 10–20                            | 15–20                              |
|         | Brain (midbrain)               | Healthy  | 25                     | 6–8                              | 15–20                              |
|         |                                |  | 16                     | 2–6                              | 10–15                              |
|         |                                |  | 10                     | 1–2                              | 5–10                               |
|         | Brain (cerebellum)             | Healthy  | 25                     | 6–8                              | 15–20                              |
|         | Brain (brainstem)              | Healthy  | 25                     | 6–8                              | 15–20                              |
|         | Spleen                         | Healthy  | 25                     | 8–10                             | 15–20                              |
|         | Kidney                         | Healthy  | 25                     | 8–10                             | 30–45                              |
|         | Small intestine                | Healthy  | 25                     | 6–8                              | 8–10                               |
|         | E16 embryo                     | Healthy  | 25                     | 2–3                              | 8–10                               |
| Human   | Tonsil                         | Inflammation/hyperplasia   | 10                     | 1–2                              | 1–5                                |
|         |                                |  | 25                     | 3–6                              | 3–8                                |
|         | Breast cancer                  | Invasive ductal carcinoma with micropapillary features<br><br>IIIB, T4aN2a | 25                     | 5–10                             | 20                                 |
|         |                                |  |                        |                                  |                                    |
|         | Glioblastoma (temporal region) | IDT WT   | 25                     | 5–7                              | 10                                 |
|         | Non-small cell lung cancer     | Squamous cell carcinoma  | 10                     | 1–3                              | 5                                  |
| 25      |                                |  | 3–5                    | 5                                |                                    |

**Table 2. Observed warm water bath incubation and drying times for internally tested tissues.** Nuclease-free warm water bath was set at 40°C. Tissue slides were dried vertically at room temperature with a small desk fan constantly blowing air.

20. If collecting additional tissue section replicates, repeat Steps 13–19 until all sections are collected.

**TIP:** Adjacent sections can be collected after this step by setting the microtome thickness to 5–10 µm. Proceed to section tissue and collect on slides. Monitor the flattening and drying times of the adjacent sections as the incubation times will be shorter due to the thinner sections.

**TIP:** Collecting multiple sections is considered good practice primarily to ensure complete tissue representation. More importantly, it provides contingency and flexibility for tissue optimization and execution of the Trekker FX workflow.

21. Once all the Tissue slides have been fully dried, place the slides in a 40°C oven and allow to dry for 12–24 hr.  
 22. After drying, place in a sealed slide mailer and store in a desiccator for up to 1 month until use. For sensitive or human samples, store the Tissue slides in a desiccator at 4°C.

**TIP:** Contact technical support ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)) to inquire about tissue compatibility with the Trekker FX assay. Additional information regarding tissue processing, sectioning, and staining can be found in the Leica Knowledge Pathway (<https://www.leicabiosystems.com/us/knowledge-pathway/>).

### C. (Optional) Adjacent Section H&E Staining

1. Identify and use the adjacent section closest to the section that would be used in the Trekker FX workflow.
2. Perform deparaffinization following steps in VII.A.
3. Fill Coplin jars or staining vessels with sufficient H&E staining and post-staining reagents to cover the tissue on the slides.
4. Proceed to H&E staining with the following protocol:

#### H&E Staining and Permanent Mounting Protocol

| Container | Reagent              | Time                   |
|-----------|----------------------|------------------------|
| 1         | Hematoxylin Solution | 3 min                  |
| 2         | Nuclease-free water  | Dipping 20x (variable) |
| 3         | Nuclease-free water  | Dipping 20x (variable) |
| 4         | Bluing Buffer        | 1 min                  |
| 5         | Nuclease-free water  | Dipping 20x (variable) |
| 6         | Eosin solution       | 1 min                  |
| 7         | Nuclease-free water  | Dipping 20x (variable) |
| 8         | 95% ethanol          | 30 sec                 |
| 9         | 100% ethanol         | 1–2 min                |
| 10        | 100% ethanol         | 1–2 min                |
| 11        | Histo-Clear          | 1–2 min                |
| 12        | Histo-Clear          | 1–2 min                |

**IMPORTANT:** Modify the protocol appropriately to generate the desired staining intensity depending on the tissue type, composition, and section thickness.



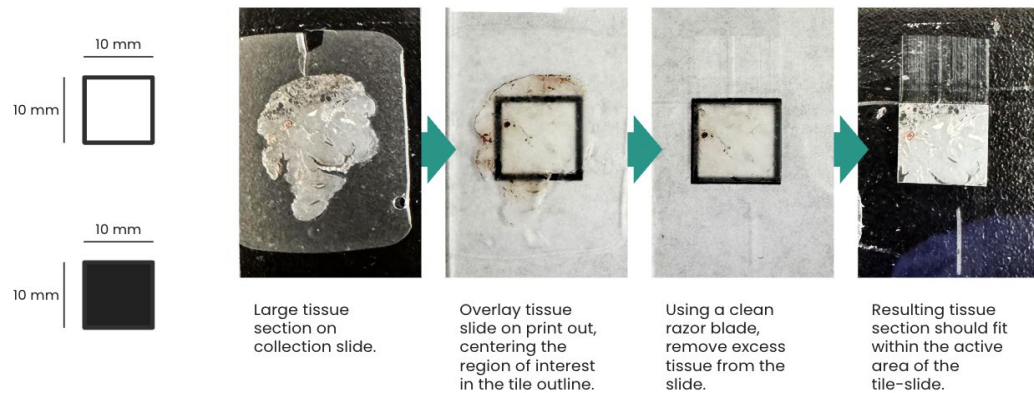
5. Remove the Tissue slide from the Coplin jar and dry the back of the slide with a Kimwipe without touching the tissue.
6. Apply the permanent mounting media on the tissue without introducing bubbles.
7. Carefully apply the coverslip at an angle without introducing bubbles.
8. Allow the mounting media to cure and dry prior to imaging.
9. After imaging, store the slide in a slide holder and store in the dark at room temperature.

## VII. Protocol

The following section provides a step-by-step protocol on how to execute the Trekker FX assay. If new to the workflow or working with a new tissue type, performing a training run on your tissue type is recommended to ensure familiarization with the protocol and successful nuclei isolation from the sample slide. For the training run, use the 10x10 Trekker FX Training Tiles (TFTB004) and follow protocol sections A through F, but skip the addition of RNase Inhibitor to the buffers unless the isolated nuclei will be used for downstream snRNA-seq. Training slides are non-functional and will not provide any spatial information if sequenced. Prior to performing the assay, it is highly recommended to be familiar with the assembly and disassembly of the Trekker Alignment Fixture (Section V. [Alignment Fixture Assembly and Disassembly](#)).

## A. Sample Deparaffinization

1. Prepare fresh reagents to be used for deparaffinization in Step 8 below.
2. Set the heat block or bead bath to 70°C for decrosslinking.
3. Pre-chill swing bucket centrifuge to 4°C and set the centrifugation speed to 800g.
4. Set the thermomixer to 37°C at 850 rpm.
5. Following tissue sectioning (from Section VI. [Tissue and Section Preparation](#)), remove the Tissue slide from the desiccator from step VI.B.22. If stored in 4°C, allow the Tissue slide to acclimate to room temperature for >2 min.
6. Ensure that the region of interest fits within the 10x10 mm active area of the Trekker FX Tile by placing it over the reference squares in Figure 13 below.
7. If the tissue is larger than the 10x10 mm area, proceed to scrape the excess tissue with a clean single-edge razor blade. Angle the razor blade at a 45-degree angle and firmly press on the slide without leaving a gap.



**Figure 13. Outline of the Trekker FX active area.** Using a printout of this page, place the Tissue slide on either the white or black box (whichever box provides the greatest contrast). If the tissue is larger than the 10x10 active area, center the region of interest and proceed to trim excess tissue outside of the outline.

**IMPORTANT:** It is critical to trim the excess tissue from the Tissue slide prior to deparaffinization to maximize the yield of spatially tagged nuclei within the final preparation.

**IMPORTANT:** When handling multiple samples or tissue types, use a new and clean single-edge razor blade for each sample to prevent potential cross contamination of analytes and spatial tags.

8. Proceed to deparaffinization with the following protocol using 30–50 ml of each solution:

### Deparaffinization Protocol


| Container | Reagent                            | Time (min) |
|-----------|------------------------------------|------------|
| 1         | Histo-Clear                        | 3          |
| 2         | Histo-Clear                        | 3          |
| 3         | 50/50 Histo-Clear and 100% ethanol | 3          |
| 4         | 100% ethanol                       | 3          |
| 5         | 100% ethanol                       | 3          |
| 6         | 90% ethanol                        | 3          |
| 7         | 70% ethanol                        | 3          |
| 8         | 50% ethanol                        | 3          |
| 9         | Nuclease-free water                | 3          |
| 10        | Nuclease-free water                | 3          |
| 11        | Nuclease-free water                | Hold       |



**IMPORTANT:** Prior to executing the Trekker FX assay, verify the section and the surrounding area is fully deparaffinized and free of paraffin. Insufficient deparaffinization can be determined by inconsistent or patchy H&E staining. If necessary, incorporate an additional Histo-Clear step and/or extend the Histo-Clear incubation to 5–10 min.

- During deparaffinization, verify that the heat block or bead bath is set to 70°C.
- Prepare Decrosslinking Solution in a 15 ml centrifuge tube by following the table below:

#### Decrosslinking Solution

| Component   | 1 sample (ml) |
|---|---------------|
|  DX Buffer | 0.14          |
| Nuclease-free water   | 13.86         |
| <b>Total</b>  | <b>14.0</b>   |

- Briefly vortex mix and gently tap on the bench surface to pop and dislodge any bubbles.
- Reserve 500 µl of Decrosslinking Solution for each sample/tile being used and store at room temperature.
- Transfer 13 ml of the Decrosslinking Solution into the provided Screw-top Slide Mailer and place in the heat block or bead bath set at 70°C (Figure 14).



**IMPORTANT:** Depending on the model of heat block or bead bath, it may take considerable time for the instrument and solution to warm up. Prepare the Decrosslinking Solution and incubate at 70°C for at least 30 min prior to the decrosslinking step to reach temperature.

- Upon completion of deparaffinization, remove the Tissue slide from the nuclease-free water and dry the slide with a Kimwipe without touching the tissue.
- Identify the tissue side of the slide(s) and lay the slide(s) on a flat surface ensuring that the tissue side is facing upwards.
- Proceed to Step B: (OPTIONAL) Same Section H&E Staining, otherwise proceed to Step C: Sample Decrosslinking for non-H&E-stained tissue sections.

## B. (OPTIONAL) Same Section H&E Staining

The following section provides an optional step-by-step protocol to perform same-section H&E staining. Performing H&E staining may result in extended dissociation times, and impact gene sensitivity and positional assignment. Inclusion of the same-section H&E staining will increase the overall assay time due to added slide imaging. It is highly recommended that the dissociation times be optimized with the Trekker FX 10x10 Training Kit prior to executing the full assay.



**IMPORTANT:** Prior to executing the Trekker FX assay with H&E staining, verify the imaging system, settings, magnification, and overall imaging times. Imaging times will vary depending on the imaging system and objective magnification being used. The protocol has been validated for up to 2 hr of imaging time without impacting nuclei yields.



**IMPORTANT:** Chemical exposure to the H&E stain and the added time for imaging the tissue can lead to ~10% reduction in gene expression and spatial tagging performance. If your project requires maximum sensitivity for low-abundance transcripts, prioritize use of adjacent sections for H&E staining and imaging, or validate the protocol before performing additional experiments.

1. Fill Coplin jars or staining vessels with sufficient fresh H&E staining reagents to cover the tissue on the slides.
2. Proceed to H&E staining with the following protocol:

### H&E Staining Protocol

| Container | Reagent              | Time (min)             |
|-----------|----------------------|------------------------|
| 1         | Hematoxylin Solution | 3                      |
| 2         | Nuclease-free water  | Dipping 20x (variable) |
| 3         | Nuclease-free water  | Dipping 20x (variable) |
| 4         | Bluing Buffer        | 1                      |
| 5         | Nuclease-free water  | Dipping 20x (variable) |
| 6         | Eosin Solution       | 1                      |
| 7         | Nuclease-free water  | Dipping 20x (variable) |

3. After staining, wipe off any excess water from the slide without touching the tissue.
4. Gently add 150–250 µl of 50% glycerol directly on the Tissue slide, avoiding bubble formation and adding enough to cover the entire tissue.
5. Coverslip the Tissue slide and gently wick off any excess glycerol.
6. Proceed immediately to imaging the tissue.
7. Fill a clean Coplin jar with nuclease-free water, enough to submerge the Tissue slide.
8. After imaging, place the Tissue slide in the Coplin jar with nuclease-free water until the coverslip comes off the Tissue slide.
9. Once the coverslip has fallen off, gently dip the Tissue slide 10x to remove any excess glycerol.
10. Identify the tissue side of the slide(s) and dry the slide with a Kimwipe without touching the tissue. Lay the slide(s) on a flat surface ensuring that the tissue side is facing upwards.
11. Add 500 µl of 0.1 N HCl to destain the tissue section.
12. Incubate for 1 min at room temperature.
13. With a clean Kimwipe, gently tilt the Tissue slide and wick off the solution.

14. Submerge the Tissue slide in a clean Coplin jar with nuclease-free water to remove residual HCl from the tissue.
15. Proceed to Step C: Sample Decrosslinking.

### C. Sample Decrosslinking

1. Gently pipette 500 µl of room temperature Decrosslinking Solution on the side(s) until tissue is completely covered (Figure 14). Minimize pipetting directly onto the tissue to prevent potential tissue detachment from the slide.
2. Incubate for 1 min at room temperature.
3. Discard the Decrosslinking Solution by tilting the slide(s) on a Kimwipe, wicking the solution off the slide(s).
4. Place the slide(s) in the screw-top slide mailer with the pre-heated Decrosslinking Solution (Figure 14), and seal.

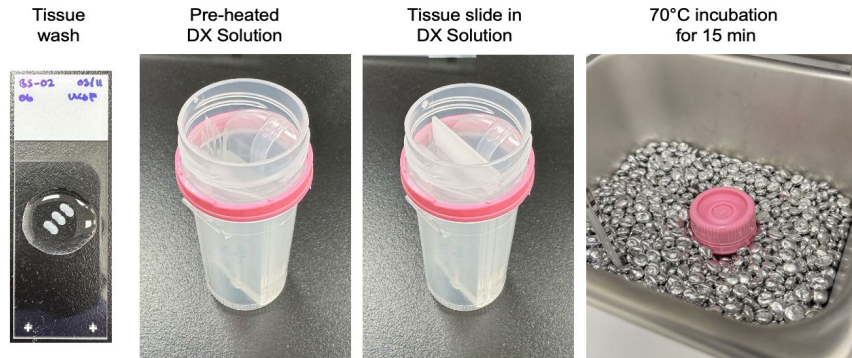


Figure 14. Decrosslinking solution on tissue and placement of the slides into the Screw-top slide mailer.

5. Incubate the assembly for 15 min at 70°C.
6. During incubation, equilibrate the Trekker FX 10x10 Tile (TF006) to room temperature and prepare the Trekker Alignment Fixture (MEC011) by installing the provided double-sided adhesive on the Bottom Plate.
7. Record the Tile ID (ex: F0009\_013) of the Trekker FX Tile label (Figure 15).



**IMPORTANT:** Each Trekker FX 10x10 Tile is unique. The Tile ID is required to retrieve the correct file for spatial barcode mapping of the sequencing data. When possible, retain the used Trekker FX 10x10 Tile until analysis is complete.



**IMPORTANT:** When using the Trekker FX 10x10 Training Kit (SK024), use a Trekker FX 10x10 Training Tile (TFT004). Trekker FX 10x10 Training Tiles can be reused multiple times.

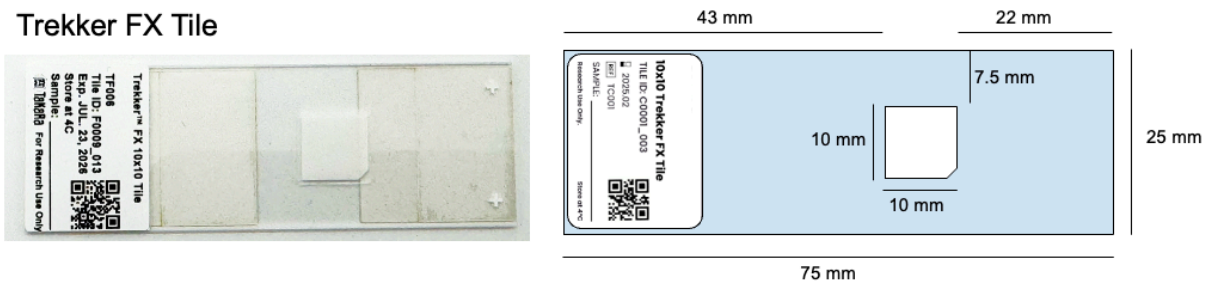


Figure 15. Representative image (left) and diagram (right) of the Trekker FX 10x10 Tile depicting the dimension and positioning of the 10 mm x 10 mm active area.



**IMPORTANT:** If there is visible damage, distortions, or large empty patches on the Trekker FX 10x10 Tile, **DO NOT** use the Tile. Note the tile ID, take a picture of the Tile, and contact technical support ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)).



**IMPORTANT:** Prior to performing the Trekker FX assay, determine the ideal sectioning thickness as this dictates the Tile-slide spacer to be used.



**TIP:** During decrosslinking incubation, the user can save time by preparing the Cleavage Solution, Stabilization Solution, Trekker Solution A, and Trekker Solution B in advance.

8. Following decrosslinking incubation, remove the Screw-top Slide Mailer from the bead bath or water bath and allow it to cool at room temperature for 1 min.
9. Remove the Tissue slide from the Screw-top Slide Mailer, and dry slide with a Kimwipe without touching the tissue.
10. Identify the tissue side of the slide(s) and lay the slide(s) on a flat surface ensuring that the tissue side is facing upwards.
11. Gently pipette 300 µl of chilled Nuclei Wash Buffer onto the slide(s) until tissue is covered.
12. Proceed immediately to Step D: Alignment Fixture Assembly and UV Cleavage.

## D. Alignment Fixture Assembly and UV Cleavage

1. Prepare the Cleavage Solution by following the table below. Briefly vortex. Tap on the bench surface and centrifuge for 2 min at 2,000 rpm to dislodge any bubbles that may have formed in solution and then store on ice.

### Cleavage Solution

| Component            | 1 sample (µl) |
|----------------------|---------------|
| ○ Nuclei Wash Buffer | 316.8         |
| 10% Tween-20         | 3.2           |
| <b>Total</b>         | <b>320</b>    |

2. Install the double-sided Slide-Fixture adhesive on the bottom plate of the Trekker Alignment Fixture for attaching the Tissue slide.
3. Take the Tissue slide, tilt, and remove the solution with a Kimwipe without touching the tissue.
4. Install the Tissue slide on the Bottom Plate on top of the Slide-Fixture adhesive centering the tissue within the fiducial markings and pressing firmly on the sides (See [Alignment Fixture Assembly and Disassembly](#)).



**TIP:** The bottom plate of the Trekker Alignment Fixture allows for some rotational freedom for Tissue slide placement. Orient and install the Tissue slide in such a way as to maximize overlap with the Trekker FX Tile. If necessary, the tissue orientation can be determined prior to trimming and starting the protocol.



**IMPORTANT:** When installing the double-sided adhesive, verify that there are no folds or creases and that both slides are flush against their respective plates. Distortions or differences in height may impact efficiency of spatial-tagging or cause tissue detachment.

5. After attaching the Tissue slide to the Bottom Plate of the Trekker Alignment Fixture, install the appropriately sized spacers on top of the Tissue slide without touching the tissue, and thread through the guide pins. Use Tile-slide spacer A for tissue sections 16–25  $\mu\text{m}$  thick. Use Tile-slide spacer B for tissue sections 10–15  $\mu\text{m}$  thick. (See [Alignment Fixture Assembly and Disassembly](#)).
6. Pipette 7.5  $\mu\text{l}$  of the Cleavage Solution on the tissue. **DO NOT** depress the pipette to the second stop to dispel the remaining solution, as this may generate unwanted bubbles on the surface.
7. Prime the Trekker FX 10x10 Tile by pipetting 300  $\mu\text{l}$  of Cleavage Solution onto the tile, completely covering the tile.
8. Incubate for 1 min at room temperature.
9. With a clean Kimwipe, tilt the Trekker FX 10x10 Tile slide and wick off excess Cleavage Solution without touching the tile.
10. Install the primed Trekker FX 10x10 Tile on the top plate of the Trekker Alignment Fixture by removing the adhesive backing, centering the tile within the fiducial markings, and firmly pressing on the sides (See [Alignment Fixture Assembly and Disassembly](#)).
11. Pipette 7.5  $\mu\text{l}$  of the Cleavage Solution on the active area of the Trekker FX 10x10 Tile. **DO NOT** depress the pipette to the second stop to dispel the remaining solution, as this may generate unwanted bubbles on the surface.



**IMPORTANT:** If visible bubbles are present on the surface of the tissue or tile, pop using a pipette tip without touching the surface. Alternatively, gently wick and reapply the Cleavage Solution.

12. Complete the assembly of the Trekker Alignment Fixture (See [Alignment Fixture Assembly and Disassembly](#)), ensuring the Trekker FX 10x10 Tile and tissue are overlapping and aligned.
13. Slowly tighten the assembly with the Tightening Ring ensuring that the Cleavage Buffer fully covers the tissue and tile, and the arrow marker of the Tightening Ring passes the arrow marker of the Top Plate.



**IMPORTANT:** If bubbles are present between the tissue and Trekker FX 10x10 Tile, **DO NOT** proceed with UV cleavage as this may lead to improper or inefficient spatial tagging. Disassemble and reassemble until no bubbles are present. Alternatively, gently wick and reapply the Cleavage Solution.

**TIP:** If the Trekker FX 10x10 Tile and Tissue slide are not aligned, the fixture can be disassembled, and the slides repositioned to target the region of interest. Spatial tagging does not happen until UV cleavage occurs.

14. Connect the UV lamp to the driver and power source and insert the lamp into the lamp holder. Set the UV meter to the max current limit (1.2 A) and max power (Figure 16, Panel C). The small screwdriver provided with the lamp can be used to set the current limit.



**IMPORTANT:** Wear protective UV glasses when working with the UV lamp. **DO NOT** look directly at the bulb while the UV lamp is on.



**Figure 16. UV lamp for the Trekker Alignment Fixture.** **Panel A.** Diagram depicting the placement of the UV lamp on the assembled Trekker Alignment Fixture. **Panel B.** The UV lamp should fit securely within the bevel of the fixture’s Tightening Ring. **Panel C.** Prior to operation, the UV meter should be set at 1.2 A. **Panel D.** The UV lamp is turned on by flipping the switch to the left to (CW).

15. Assemble the UV Lamp and Trekker Alignment Fixture (Figure 16, Panels A–B).
16. Turn on the UV Lamp by moving the switch to the left (to the position labeled CW) (Figure 16, Panel D) and set a timer for 60 sec.
17. After 60 sec, turn off (by moving the switch to the center position, labeled TRIG) and remove the UV Lamp.
18. Incubate the post-cleaved Trekker Alignment Fixture at room temperature for 7.5 min on a flat surface.
19. During incubation, prepare fresh Stabilization Solution by following the table below. Briefly vortex, centrifuge, and store on ice (Stabilization Solution will be used for Step F.1):

**Stabilization Solution**

| Component                             | 1 sample (µl) |
|---------------------------------------|---------------|
| ○ Nuclei Wash Buffer                  | 700           |
| 32% Paraformaldehyde Aqueous Solution | 100           |
| <b>Total</b>                          | <b>800</b>    |

20. After the 7.5 min of incubation, carefully disassemble the Trekker Alignment Fixture without touching the Trekker FX 10x10 tile or the tissue (see [Alignment Fixture Assembly and Disassembly](#)).
21. Remove the spatially tagged Tissue slide from the Trekker Alignment Fixture and lay on a flat clean surface.
22. Carefully add 300 µl of chilled Nuclei Wash Buffer to the surface of the tissue.
23. Incubate for 30 sec at room temperature.
24. With a clean Kimwipe, tilt the Tissue slide and wick off the Nuclei Wash Buffer.
25. Carefully add 300 µl of chilled Nuclei Wash Buffer to the surface of the tissue.
26. Incubate for 30 sec at room temperature.
27. With a clean Kimwipe, tilt the Tissue slide and wick off the Nuclei Wash Buffer.

28. Gently pipette 300 µl of the chilled Stabilization Solution to the surface of the tissue. Keep the remainder of the Stabilization Solution on ice and save for Step 4.1 of the Trekker FX workflow.
29. Incubate sample for 5 min at room temperature.
30. With a clean Kimwipe, tilt the Tissue slide and wick off the Stabilization Solution.
31. Add 300 µl of chilled Nuclei Wash Buffer to the surface of the tissue.
32. Incubate for 30 sec at room temperature.
33. With a clean Kimwipe, tilt the Tissue slide and wick off the Nuclei Wash Buffer.
34. Add 300 µl of chilled Nuclei Wash Buffer to the surface of the tissue.
35. Incubate for a minimum of 30 sec at room temperature.
36. Immediately proceed to Step E: Nuclei Isolation.



**IMPORTANT:** If partial tissue detachment onto the Trekker tile is observed, recovery can be performed by pipetting 100 µl of Trekker Solution A onto the Trekker tile and pipette mixing to dislodge and recover the detached tissue into the Protein LoBind collection tube. If the tissue or a significant amount of the tissue (>10%) comes off the sample slide, **DO NOT** proceed. Take a picture of the tile and tissue slide and email it to technical support ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)).

**IMPORTANT: DO NOT** immediately discard the used Trekker FX 10x10 tile. Keep the tile for reference or until analysis is complete, in case additional troubleshooting is required.

## E. Nuclei Isolation

1. Pre-chill swing bucket centrifuge to 4°C and set the centrifugation speed to 800g.
2. Set the thermomixer to 37°C at 850 rpm.
3. Clean single-edge razor blade by spraying all surfaces with 70% ethanol and carefully wiping dry with Kimwipe prior to use. Perform proper contamination control by using a new single-edge razor blade for each sample.
4. Resuspend fresh TD Enzyme with 1,000 µl of nuclease-free water and place it on ice.
5. Prepare fresh Trekker Solution A by following the table below in a new Protein LoBind 1.5 ml centrifuge tube, then pipette to mix (15x), centrifuge, and store on ice:

### Trekker Solution A

| Component                  | 1 sample (µl) |
|----------------------------|---------------|
| ○ Nuclei Isolation Buffer  | 785           |
| ○ TD Enzyme (resuspended)* | 200           |
| ○ RNase Inhibitor**        | 15            |
| <b>Total</b>               | <b>1,000</b>  |

\*For unused resuspended TD Enzyme, make 230 µl aliquots and store at –20°C. Use the remaining TD Enzyme within 2 months of resuspension. Thaw on ice when using resuspended aliquots.

\*\*For training and tissue optimization, exclude RNase Inhibitor from the formulation and replace with an equal volume of Nuclei Isolation Buffer.

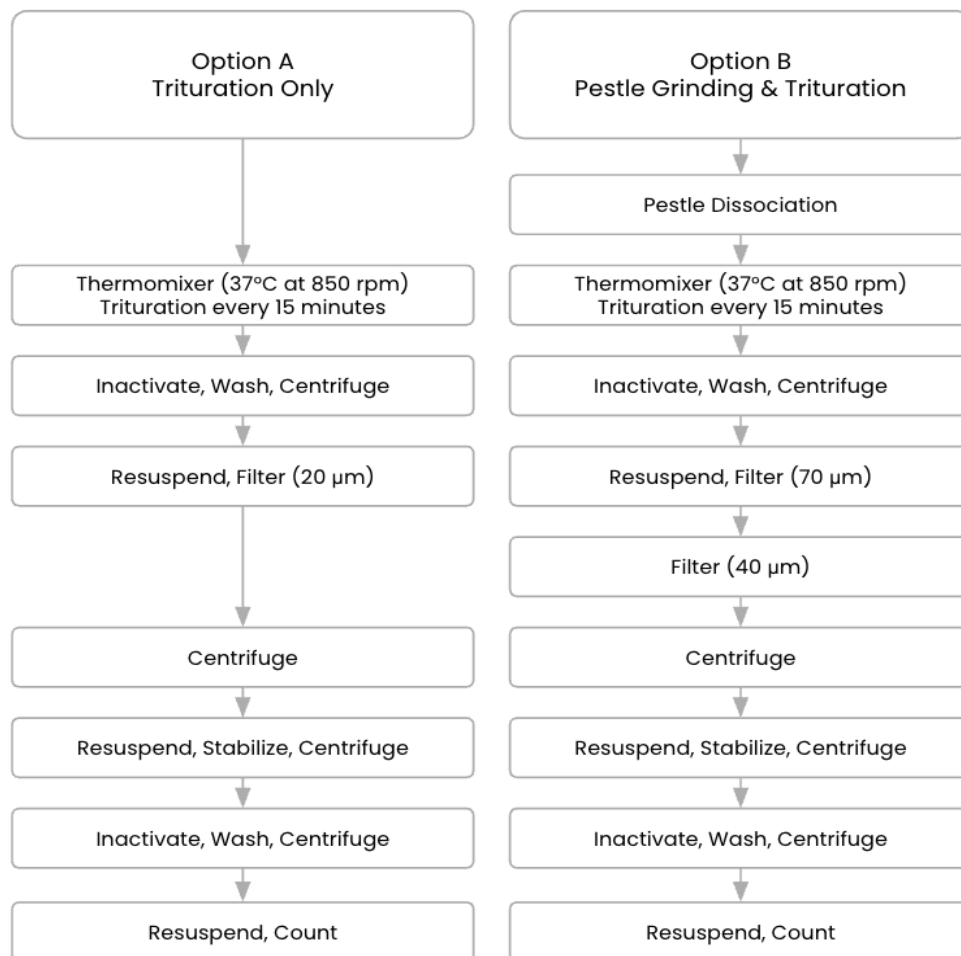
6. Prepare fresh Trekker Solution B by following the table below in a new Protein LoBind 5 ml centrifuge tube, then pipette to mix (15x), centrifuge, and store on ice:

**Trekker Solution B**

| Component            | 1 sample (µl) |
|----------------------|---------------|
| ○ Nuclei Wash Buffer | 3,523         |
| ● BSA-H              | 124.5         |
| ○ RNase Inhibitor*   | 52.5          |
| <b>Total</b>         | <b>3,700</b>  |

\*For training and tissue optimization, exclude RNase Inhibitor from the formulation and replace with an equal volume of Nuclei Wash Buffer.

- Proceed with either Step E.8 Option A: Trituration Only, or Step E.9 Option B: Pestle Grinding and Trituration tissue dissociation workflow (Figure 17). Depending on the tissue type and composition, some samples may require additional mechanical dissociation. For these difficult to dissociate tissues, proceed with Option B for pestle grinding and trituration. Examples of difficult to dissociate tissues include human tissues and highly fibrotic tissues, such as skin, fibrotic tumors, dense connective tissues, calcified tissues, muscle, heart, or scarred tissues.



**Figure 17. Side-by-side workflows for tissue dissociation and nuclei isolation.** Option A: Trituration Only and Option B: Pestle Grinding & Trituration.

**8. Option A: Trituration Only**

1. Using a P200 pipette, aspirate 100 µl of Trekker Solution A (isolation solution) and set aside. This will be used to dislodge the tissue from the single-edge razor blade.
2. With a clean Kimwipe, tilt the Tissue slide and wick off the Nuclei Wash Buffer from the slide.
3. Using the cleaned single-edge razor blade, press firmly on the slide at a ~45-degree angle and then scrape the tissue off the slide. Pipette tissue off the razor blade with 100 µl of Trekker Solution A and into a labeled 1.5 ml Eppendorf Protein LoBind tube (tube #1).
4. Add 900 µl of Trekker Solution A to the tube.
5. Using a P1000 pipette set to 700 µl volume, carefully triturate the tissue in the Trekker Solution A by pipette mixing 20–30x. During trituration, avoid fully depressing the piston and generating excessive bubbles.
6. Incubate in a pre-heated thermomixer for a total of 60–90 min at 37°C.
7. Every 15 min, pipette mix (triturate) the sample 20–30x to further mechanically dissociate the sample.



**IMPORTANT:** The total dissociation time will vary between tissue types. It is important to perform tissue optimization with the Trekker FX Training Kit to determine the ideal time for your sample. During optimization or when necessary, check the nuclei quality using a microscope after each trituration round. Failure to fully dissociate tissue or over-lysis may lead to reduced nuclei yield and loss of tissue regional representation.

8. After dissociation, immediately place the tube on ice and incubate for 1 min.
9. Pipette mix 10x and transfer 500 µl of sample into a second 1.5 ml Protein LoBind tube (tube #2).
10. Drop by drop, add 1,000 µl of Trekker Solution B into each of the two sample tubes, followed by gently pipette mixing 5x.
11. Spin the tubes down in the pre-chilled swing bucket centrifuge set to 4°C at 800g for 5 min.
12. Carefully remove the tubes from the centrifuge and immediately place them on ice.
13. Use a P1000 pipette to remove the supernatant from both tubes, being careful not to disturb the pellet. The pellet may not be visible. Leave behind approximately 50–70 µl of solution.
14. Resuspend tube #1 of isolated nuclei in 800 µl of Trekker Solution B. Gently pipette mix 5x to resuspend the sample.
15. Set pipette to 1,000 µl volume, transfer tube #1 contents to tube #2 to combine the isolated nuclei. **DO NOT** discard the tubes. Gently pipette mix 5x.
16. In a new 1.5 ml Protein LoBind centrifuge tube (tube #3), place a pre-chilled pluriStrainer Mini 20 µm cell strainer.
17. Aspirate the resuspended nuclei and slowly pass through the strainer into collection tube #3. **DO NOT** discard the strainer or tubes.
18. Wash tube #1 with 350 µl of Trekker Solution B. Set pipette to 500 µl volume and transfer tube #1 contents to tube #2 to combine the isolated nuclei.
19. Pipette remaining solution through the pluriStrainer Mini 20 µm cell strainer into collection tube #3. Keep the filtrate and discard tube #1 and tube #2. If necessary, use a P1000 pipette to aspirate and collect any remaining solution from the bottom of the filter prior to disposal.
20. After filtration, spin collection tube #3 down in the pre-chilled swing bucket centrifuge set to 4°C at 800g for 5 min.
21. Use a P1000 pipette to remove the supernatant from the tube, being careful not to disturb the pellet. The pellet may not be visible. Leave behind 50–70 µl of buffer, then proceed to Step F: Nuclei Stabilization.



**IMPORTANT:** Depending on the tissue type and species, larger filter pore sizes (such as the pluriStrainer Mini 40 µm cell strainer) may be used to minimize filter clogging and maximize nuclei yield.

9. Option B: Pestle Grinding and Trituration

1. Using a P200 pipette, aspirate 100 µl of Trekker Solution A and set aside. This will be used to dislodge the tissue from the single-edge razor blade.
2. With a clean Kimwipe, tilt the Tissue slide and wick off the Nuclei Wash Solution from the slide.
3. Using the cleaned single-edge razor blade, scrape the tissue off the slide. Pipette the tissue off the razor blade with 100 µl of Trekker Solution A into a 1.5 ml Eppendorf Protein LoBind tube.
4. Using a new and clean pestle, grind the tissue by pressing down and twisting.
5. Repeat pestle grinding up to 15–25x.



**IMPORTANT: DO NOT** exceed 1 min of pestle grinding. Excessive pestle grinding or prolonged grinding can cause over-lysis and sample clumping, leading to reduced nuclei yield and compromised quality.

6. Wash the pestle with the remaining 900 µl of Trekker Solution A.
7. Using a P1000 pipette set to 700 µl volume, carefully triturate the tissue in the Trekker Solution A by pipette mixing 20–30x. During trituration, avoid fully depressing the piston and generating excessive bubbles.
8. Incubate in a pre-heated thermomixer for a total of 60–90 min at 37°C.
9. Every 15 min, pipette mix (triturate) the sample 20–30x to further mechanically dissociate the sample.



**IMPORTANT:** The total dissociation time will vary between tissue types. It is important to perform tissue optimization with the Trekker FX Training Kit to determine the ideal time for your sample. During optimization or when necessary, check the nuclei quality using a microscope after each trituration round. Failure to fully dissociate tissue or over-lysis may lead to reduced nuclei yield and loss of tissue regional representation.

10. After dissociation, immediately place the tube on ice and incubate for 1 min.
11. Pipette mix 10x and transfer 500 µl of sample into a second 1.5 ml Protein LoBind tube (tube #2).
12. Drop by drop, add 1,000 µl of Trekker Solution B into each of the two sample tubes, followed by gently pipette mixing 5x.
13. Spin the tubes down in the pre-chilled swing bucket centrifuge set to 4°C at 800g for 5 min.
14. Carefully remove the tubes from the centrifuge and immediately place them on ice.
15. Use a P1000 pipette to remove the supernatant from both tubes, being careful not to disturb the pellet. The pellet may not be visible. Leave behind approximately 50–70 µl of solution.
16. Resuspend tube #1 of isolated nuclei in 800 µl of Trekker Solution B. Gently pipette mix 5x to resuspend the sample.
17. Set pipette to 1,000 µl volume, transfer tube #1 contents to tube #2 to combine the isolated nuclei. **DO NOT** discard the tubes. Gently pipette mix 5x.
18. In a new 1.5 ml Protein LoBind centrifuge tube (tube #3), place a pre-chilled pluriStrainer Mini 70 µm cell strainer.
19. Aspirate the resuspended nuclei and slowly pass through the strainer into collection tube #3. **DO NOT** discard the strainer or tubes.
20. Wash tube #1 with 350 µl of Trekker Solution B. Set pipette to 500 µl volume and transfer tube #1 contents to tube #2 to combine the isolated nuclei.

21. Pipette remaining solution through the pluriStrainer Mini 70 µm cell strainer into collection tube #3. Keep the filtrate and discard tube #1 and tube #2. If necessary, use a P1000 pipette to aspirate and collect any remaining solution from the bottom of the filter prior to disposal.
22. In a new 1.5 ml Protein LoBind centrifuge tube (tube #4), place a pre-chilled pluriStrainer Mini 40 µm cell strainer. Aspirate all the filtrate nuclei and slowly pass through the strainer. If necessary, use a P1000 pipette to aspirate and collect any remaining solution from the bottom of the filter prior to disposal.
23. After the double-filtration, spin collection tube #4 down in the pre-chilled swing bucket centrifuge set to 4°C at 800g for 5 min.
24. Use a P1000 pipette to remove the supernatant from the tube, being careful not to disturb the pellet. The pellet may not be visible. Leave behind 50–70 µl of buffer, then proceed to Step F: Nuclei Stabilization.



**IMPORTANT:** Depending on the tissue type and species, larger filter pore sizes (such as the pluriStrainer Mini 40 µm cell strainer) may be used to minimize filter clogging and maximize nuclei yield.

## F. Nuclei Stabilization

1. Drop by drop, add 500 µl of chilled Stabilization Solution from Step D.16 and resuspend by gently pipette mixing 5x.
2. Incubate for 15 min on ice.
3. Spin the tube down in the pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min.
4. Remove the supernatant, being careful not to disturb the pellet. Leave behind 50–70 µl of buffer.
5. Drop by drop, add 500 µl of Trekker Solution B and resuspend by gently pipette mixing 5x.
6. Spin the tube down in the pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min.
7. Remove the supernatant, being careful not to disturb the pellet. Leave behind 50–70 µl of buffer.
8. Using a P200 pipette set at 30 µl, gently pipette mix the nuclei suspension 5x, store on ice, and proceed to nuclei counting. If necessary, dilute a small aliquot of your sample in Trekker Solution B or the staining buffer for counting.
9. Count nuclei with AO/PI or Ethidium Homodimer-1 and dilute the nuclei to the desired concentration based on the guidelines in the [GEM-X Flex Gene Expression Reagent Kit for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide](#) (CG000789, Rev B) or [GEM-X Flex v2 with Feature Barcode technology for Protein User Guide](#) (CG000835, Rev B).

**TIP:** Counting with a fluorescent automated counter or microscope is strongly recommended. The use of Trypan Blue can lead to overestimated nuclei counts as the stain cannot differentiate between nuclei, poor quality nuclei, and debris.

10. Samples can be stored at –80°C for up to 1 month (Trekker FX Long-term Storage Protocol) or proceed immediately to the next step. If directly proceeding with the next step, the freshly dissociated samples can be placed on ice for up to 4 hrs.
11. Prepare fresh Tissue Resuspension Buffer according to the table below and place the buffer on ice:

**Tissue Resuspension Buffer\***

| Component                 | 1 Sample (µl) |
|---------------------------|---------------|
| 1X PBS                    | 248           |
| 1M Tris buffer,<br>pH 8.0 | 25            |
| 10% BSA                   | 1             |
| RNase Inhibitor           | 3             |
| Nuclease-free<br>water    | 223           |
| <b>Total</b>              | <b>500</b>    |

\*Formulation details can be found in the [Sample Preparation from FFPE Tissue Sections for GEM-X Flex Gene Expression Demonstrated Protocol](#) (CG000784, Rev A).

12. Dropwise, add 500 µl of Tissue Resuspension Buffer to each sample on ice
13. Gently pipette mix 5x and store on ice.
14. Proceed immediately to Step G: Single-Cell Capture and Library Generation.



**IMPORTANT:** Nuclei are stable for up to 5 hours on ice. Proceed to single-cell capture within the same day as isolation.

## G. Single-Cell Capture and Library Generation (GEM-X Flex v1)



**IMPORTANT:** Please verify that you are using the correct version of the 10X Genomics [GEM-X Flex Gene Expression Reagent Kit for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide](#) (CG000789, Rev B). Using the incorrect protocol version or primers may result in failed Trekker library generation.



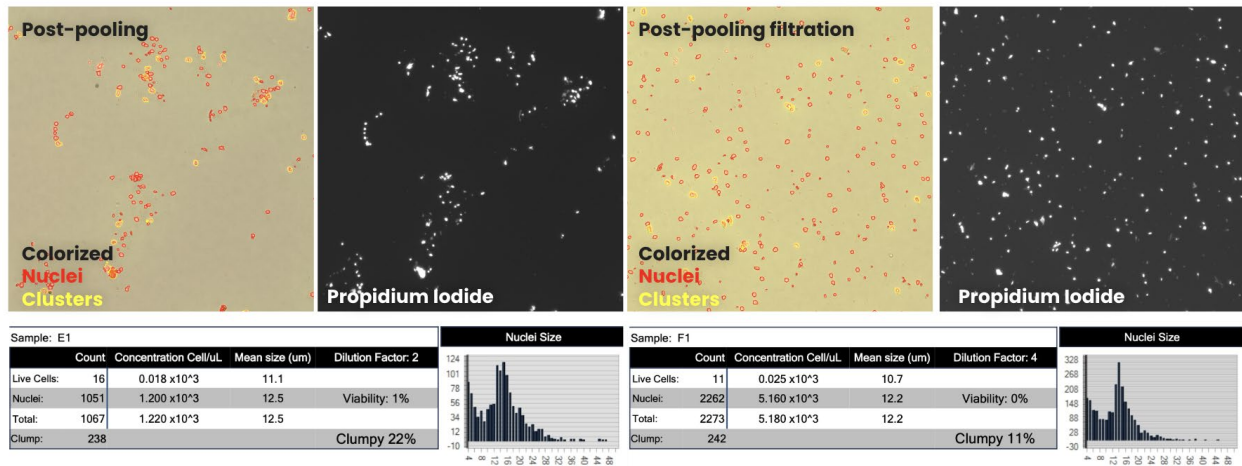
**IMPORTANT:** Follow the modification described in this section carefully. Failure to do so may result in lower nuclei recovery and/or poor library quality.

1. Verify that the number of samples match the appropriate number of multiplexing barcodes and the pooling strategy planned for the experiment.
2. Perform single nuclei capture and library prep following instructions for [GEM-X Flex Gene Expression Reagent Kit for Multiplexed Samples with Feature Barcode technology for Protein using Barcode Oligo Capture User Guide](#) (CG000789, Rev B) with the following modifications:
  1. At Step 1.1.d., centrifuge the nuclei in **Tissue Resuspension Buffer** (instead of Quenching Buffer B) in a pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min.
  2. At Step 1.1.e., leave approximately 20–25 µl when removing supernatant to prevent nuclei loss. If necessary, use a P200 pipette for more precise aspiration and maximum nuclei retention
  3. In section 2.1, perform Pooled Wash Workflow (Option A) with <50,000 cells/hyb as outlined on page 56. Follow this approach even if the starting nuclei count exceeds ≥50,000 cells/hyb to preserve the maximum number of nuclei.
  4. Skip Step 2.1.A.e.

- At [Step 2.1.A.q](#), on page 60, target for maximum recovery by resuspending the sample in 250 µl chilled Post-Hyb Resuspension Buffer B.
- At Step 2.1.A.s, count nuclei with AO/PI or Ethidium Homodimer-1 to determine nuclei concentration and clumpiness.



**IMPORTANT:** If >20% clumpiness is observed after pooling (Figure 18), then proceed to filter the sample. Add an additional 250 µl of Post-Hyb Resuspension Buffer B (~500 µl total) and pipette mix 5x. Pass the sample through a pre-chilled pluriStrainer Mini 40 µm cell strainer in a new tube. Spin the tube with the filtrate down in the pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min. Remove the supernatant, being careful not to disturb the pellet. Leave behind 50–70 µl of solution.



**Figure 18. Example of broad clump formation after pooling.** After passing through a 40 µm filter, a reduction of clumps is observed.

- DO NOT** store samples as described in step 2.1.A.u and proceed immediately to [Step 3: GEM Generation & Barcoding](#) on page 70.
- Generate the Trekker library following steps in the 10X protocol for ‘Protein Expression Library Construction’.



**IMPORTANT:** Use [Pre-Amp Primers C \(PN 2000953\)](#) during the Pre-Amplification PCR (Step 4.2). Verify the name and part number as indicated on page 85 and page 87. Using the wrong primer will result in failed Trekker library generation. If Pre-Amp Primer B (PN 2000529) was used, reach out to Technical Support ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)) for guidance.

- Quantify the Trekker libraries using Qubit (1X dsDNA HS Assay Kit) and a Bioanalyzer or TapeStation following the manufacturer’s guidelines (Bioanalyzer High Sensitivity DNA assay or TapeStation High Sensitivity D5000 assay or TapeStation D5000 assay). Select the region between 150–300 bp to determine the average size of the library. See below for an example Trekker library trace on the TapeStation (D5000):

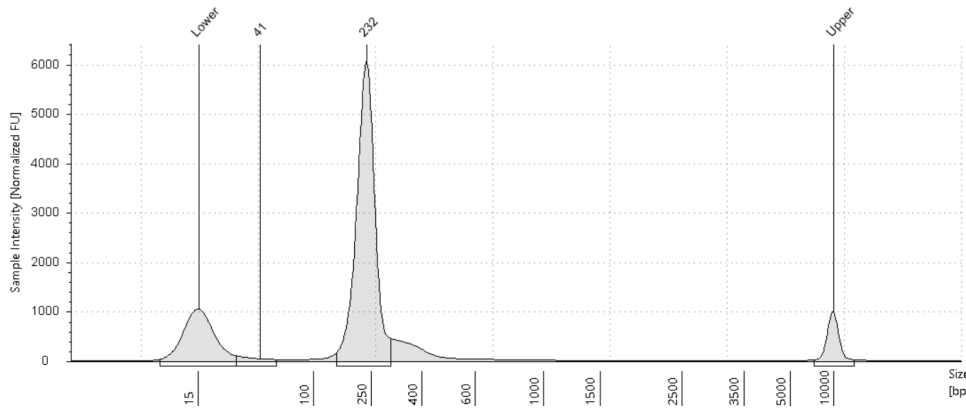


Figure 19. Example Trekker library trace from a 1:80 dilution run through a TapeStation (D5000).

## H. Single-Cell Capture and Library Generation (GEM-X Flex v2 - Apex)



**IMPORTANT:** Please verify that you are using the correct version of the 10X Genomics [GEM-X Flex v2 with Feature Barcode technology for Protein User Guide](#) (CG000835, Rev B). Using the incorrect protocol version or primers may result in failed Trekker library generation.



**IMPORTANT:** Prior to executing the single cell assay, decide on the appropriate normalization and pooling strategy with the 10x Genomics GEM-X Flex v2 Pooling Workbook (CG001698, Rev A). Normalization can be performed either before or after overnight WTA probe hybridization. For more guidance, refer to Probe Hybridization on page 34 of the User Guide.



**IMPORTANT:** Follow the modification described in this section carefully. Failure to do so may result in lower nuclei recovery and/or poor library quality.

1. Verify that the number of samples match the appropriate number of multiplexing barcodes and the pooling strategy planned for the experiment.
2. Perform single nuclei capture and library prep following instructions for [GEM-X Flex v2 with Feature Barcode technology for Protein User Guide](#) (CG000835, Rev B) with the following modifications:
  1. At Step 1.1.d., centrifuge the nuclei in **Tissue Resuspension Buffer** (instead of Quenching Buffer B) in a pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min.
  2. At Step 1.1.e., leave approximately 20–25 µl when removing supernatant to prevent nuclei loss. If necessary, use a P200 pipette for more precise aspiration and maximum nuclei retention
  3. In section 2.1 overnight probe hybridization.
  4. In section 3.1, perform Pooled Wash Workflow (Option A) with  $\leq 50,000$  cells/hyb as outlined on page 64. Follow this approach even if the starting nuclei count exceeds  $\geq 50,000$  cells/hyb to preserve the maximum number of nuclei.
  5. If performing post-hybridization pooling where the samples have been normalized prior to WTA probe hybridization, proceed with Step 3.1.A.c. If samples are unnormalized, proceed with Step 3.1.A.e. **DO NOT** perform Step 3.1.A.d.
  6. At Step 3.1.A.w, count nuclei with AO/PI or Ethidium Homodimer-1 to determine nuclei concentration and clumpiness.



**IMPORTANT:** If >20% clumpiness is observed after pooling (Figure 18), then proceed to filter the sample. Add an additional 250 µl of Post-Hyb Resuspension Buffer B (~500 µl total) and pipette mix 5x. Pass the sample through a pre-chilled pluriStrainer Mini 40 µm cell strainer in a new tube. Spin the tube with the filtrate down in the pre-chilled centrifuge with swing buckets set to 4°C at 800g for 5 min. Remove the supernatant, being careful not to disturb the pellet. Leave behind 50–70 µl of solution.

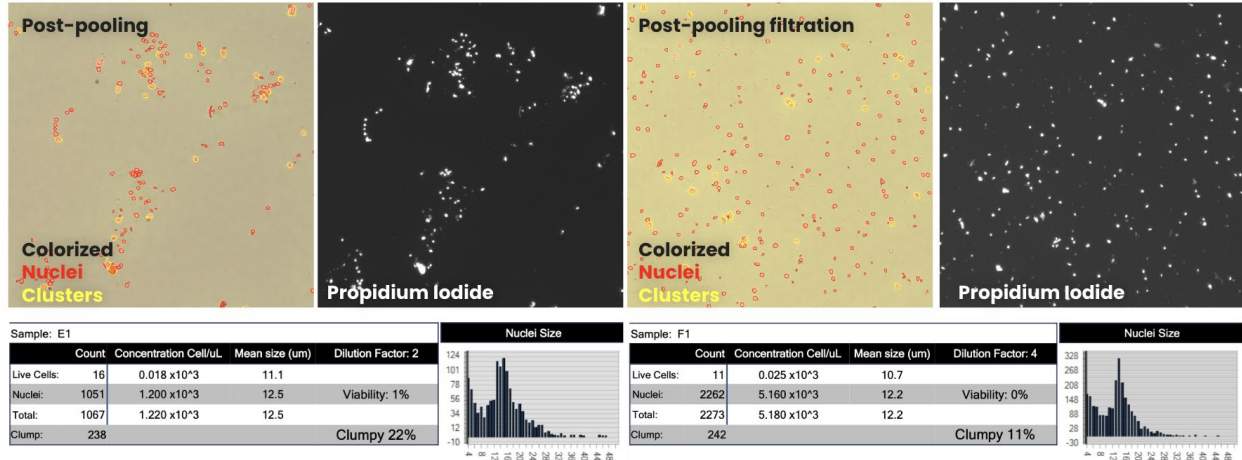


Figure 20. Example of broad clump formation after pooling. After passing through a 40 µm filter, a reduction of clumps is observed.

7. **DO NOT** store samples as described in step 2.1.A.x and proceed immediately to Step 3: GEM Generation & Barcoding on page 78.
3. Generate the Trekker library following steps in the 10X protocol for ‘Protein Expression Library Construction’.



**IMPORTANT:** Use Pre-Amp Primers C (PN 2000953) during the Pre-Amplification PCR (Step 5.2). Verify the name and part number as indicated on page 92 and page 94. Using the wrong primer will result in failed Trekker library generation. If Pre-Amp Primer B (PN 2000529) was used, reach out to Technical Support ([Technical\\_Support@takarabio.com](mailto:Technical_Support@takarabio.com)) for guidance.

1. Quantify the Trekker libraries using Qubit (1X dsDNA HS Assay Kit) and a Bioanalyzer or TapeStation following the manufacturer’s guidelines (Bioanalyzer High Sensitivity DNA assay or TapeStation High Sensitivity D5000 assay or TapeStation D5000 assay). Select the region between 150–300 bp to determine the average size of the library. See below for an example Trekker library trace on the TapeStation (D5000):

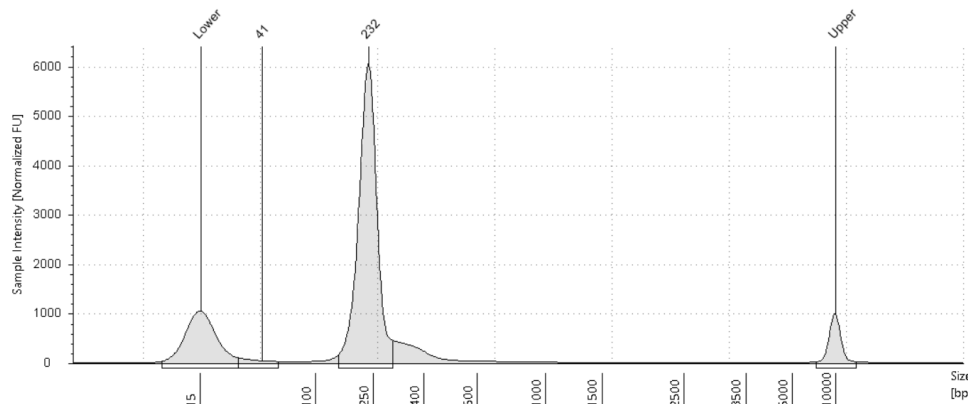


Figure 21. Example Trekker library trace from a 1:80 dilution run through a TapeStation (D5000).

## I. Sequencing Parameters

### Recommended Sequencing Configuration:

| Sequencing read | Recommended read length (bp) |
|-----------------|------------------------------|
| Read 1          | 28                           |
| i7 index        | 10                           |
| i5 index        | 10                           |
| Read 2          | 90                           |

\*For GEX (WTA) libraries, sequence a minimum of 10,000 read pairs per cell/nuclei.

\*\*For Trekker FX libraries, sequence a minimum of 2,000 read pairs per reads/nuclei.

### PhiX Spike-in Recommendations:

- If the Trekker library is sequenced with GEX (WTA) libraries, follow the sequencing recommendations in the 10x Flex User Guide for PhiX spike-in.
- If the Trekker library is sequenced on its own, additional PhiX spike-in may be needed.
  - NextSeq 1000/2000: 10% PhiX spike-in when pooling with only Trekker libraries
  - NextSeq 500/550: 10% PhiX spike-in when pooling with only Trekker libraries
  - NovaSeq 6000 and Novaseq X:
    - 5% PhiX spike-in when pooling with non-Trekker libraries
    - 10% PhiX spike-in when pooling with only Trekker libraries

## J. Data Analysis

For details on data processing and output interpretation, refer to the [Trekker Primary Analysis Pipeline](#) for local analysis, and the [Takara Bio Spatial Bioinformatics Portal](#) for cloud analysis, which can both be found at [takarabio.com](http://takarabio.com).

## VIII. Troubleshooting

| Problem  | Possible Explanation   | Solution   |
|--|--|--|
| <b>Tissue section curling during sectioning</b>    | The tissue section is not flat                                 | Use a small brush to flatten the tissue section either during or after sectioning prior to placing onto the warm water bath. Minor curling is tolerable as the warm water bath will flatten the tissue as it hydrates. Uncurling in the water bath can be guided or assisted with a small fine brush.  |
|  | Tissue block is too dry  | Incubate the FFPE block in cold nuclease-free water (or wet ice). Depending on the tissue type and processing conditions, some blocks may be too dehydrated. Submerge the block for at least 15 min or longer. This will allow partial hydration of the exposed tissue. Rehydration may need to be repeated during consecutive section collection. |
|  | Dull blade leading to nonuniform sectioning                    | Replace blade and ensure that it is free of debris.  |
|  | Inconsistent tissue processing and paraffin infiltration.      | Non-uniform processing occurs due to improper processing conditions and/or older processing reagents. Improper tissue processing can be identified by inconsistent or non-uniform H&E staining. If the problem persists, reprocess the FFPE block to obtain a more uniform wax infiltration within the tissue block.                               |
| <b>Tissue section tears during sectioning</b>      | Debris on blade  | Remove the blade and wipe with a Kimwipe with 70% ethanol to remove any debris. If the problem persists, discard the old blade and replace it with a new clean blade.  |
|  | Calcified regions or debris within the tissue                  | Rotate tissue block so that the calcified region or debris is the last to be cut.  |
| <b>Bubble formation in or around sections</b>      | Bubbles in water   | Prior to collection, dislodge bubbles at the base of the water bath by running a brush around the bottom. This is then followed by running a Kimwipe across the surface of the water to remove any debris. If the problem persists, consider degassing the water by boiling or vacuum filtration prior to use.                                     |
|  | Bubbles trapped underneath tissue in the water bath incubation | Once an air bubble is trapped underneath the tissue section in the water bath, it is difficult to remove. Consider recollecting a new section.   |
|  | Oven temperature is too high during overnight drying           | Too high of a temperature during overnight drying may lead to rapid evaporation of water trapped under the tissue section. Ensure the temperature is constant between 37–40°C.   |
| <b>Wrinkled tissue section on collection slide</b> | Uneven drying  | Use a desktop fan to allow for even drying of the Tissue slide after collecting from the water bath.   |
|  | Insufficient warm water bath incubation time                   | Insufficient warm water bath incubation may lead to uneven hydration and expansion of the section. Thicker sections will require longer incubation times compared to thinner sections.   |

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|   | Water bath temperature too low                                      | Too low of a water temperature may lead to inefficient hydration and expansion of the tissue section. Ensure that the water bath temperature is between 37–40°C.   |
|   | Dull blade leading to nonuniform sectioning                         | Replace blade. Nonuniform section thickness will result in uneven hydration and expansion times that would result in wrinkled sections.  |
|   | Inconsistent tissue processing and paraffin infiltration            | Reprocess the FFPE block to obtain a more uniform wax infiltration.  |
| <b>Cores within tissue microarrays (TMAs) detaching from the section.</b> | Over/under hydration of tissue block prior to sectioning            | Depending on the composition and the tissue types in the TMAs, pre-sectioning hydration times may vary. Certain tissue types may hydrate more readily, and the expansion may cause the cores to slightly detach from the main block. Adjust the pre-sectioning hydration time accordingly to the specific TMA and tissue types.              |
|   | Overhydration of tissue section prior to slide mounting             | Like pre-sectioning hydration, post-sectioning hydration will lead to expansion of the tissue and the surrounding paraffin. Excessive hydration and expansion will lead to the core detaching from the surrounding paraffin.   |
|   | Insufficient bonding of the cores to the recipient paraffin block   | Place the TMA on clean glass microscope slide, then place the assembly face-down in a pre-heated 60°C oven. Incubate for approximately 10 minutes to allow for the cores to sufficiently bond to the surrounding paraffin. After incubation, place the assembly directly onto a cooling block to solidify the wax and stabilize the bonding. |
|   | Individual tissue cores curling or not being sectioned properly     | Section slowly and observe the behavior of the individual cores. If necessary, gently press on the core with a brush to prevent curling. If problem persists, clean or replace the microtome blade.  |
| <b>Core biopsy tissue detaching or curling from section.</b>              | Suboptimal orientation of tissue block relative to sectioning blade | When possible, orient the tissue diagonal or perpendicular to the sectioning blade. This enables sufficient surface area and attachment points to stabilize the section during sectioning. Avoid sectioning core needle biopsies samples oriented parallel to the sectioning blade.  |
|   | Over/under hydration of tissue block prior to sectioning            | Depending on the composition of the core needle biopsy, pre-sectioning hydration times may vary. Certain tissue types may hydrate more readily, and the expansion may cause the thin tissue sample cores to slightly detach from the surrounding. Adjust the pre-sectioning hydration time accordingly to the specific tissue type.          |
| <b>Tissue detachment onto the Trekker FX 10x10 Tile</b>                   | Non-adhesive microscope slide was used for tissue sections          | FFPE tissue sections placed on non-adhesive microscope slides have higher likelihood of detachment. Verify that Superfrost Plus Microscope Slides are used for tissue sectioning and placement. Other adhesive microscope slides have not been validated.  |
|   | Inaccurate tissue sections  | Settings of the microtome may be inaccurate. Perform preventative maintenance on the microtome to ensure accurate section thickness.   |

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|                         | Tissue hydration yielded thicker sections                | Depending on type or how it was processed, some tissues may swell or over hydrate. The resulting thicker tissue section may adhere to the tile surface and lead to detachment. If tissue contact onto the surface of the tile was observed, slightly loosen the Tightening Ring to reduce contact (see Step V.A.16.).   |
|                         | Use of wrong tile-slide spacers                          | Use of the thinner Tile-slide Spacer B on sections >15 µm will result in tissue being excessively compressed onto the surface of the tile. Use the Tile-slide Spacer A for sections between 16–25 µm thickness.   |
|                         | Excessive wrinkling of the tissue                        | Excessive wrinkling will result in a non-uniform surface and tissue thickness during alignment. See above troubleshooting tips for wrinkled tissues. If the detachment impacts >10% of tissue or the region of interest, <b>DO NOT</b> proceed with protocol.   |
|                         | Insufficient tissue slide drying after collection        | The tissue may not be fully adhered onto the microscope slide. Ensure the tissue is thoroughly adhered onto the microscope slide by performing the room temperature and overnight drying.   |
|                         | Tissue is generally sticking to the Trekker tile surface | The tissue is non-specifically sticking to the Trekker tile. Ensure that the Trekker tile is properly primed and the cleavage solution properly covers the active area of the tile. If partial tissue detachment on the Trekker tile is observed, recovery can be performed by pipetting 100 µl of Trekker Solution A on the Trekker tile and pipette mixing to dislodge and recover in the Protein LoBind collection tube. |
| <b>Low nuclei yield</b> | Tissue section too thin                                  | Depending on the organ, tissue type, and tissue composition, the thinner sections may lead to partially cut nuclei and less whole nuclei. Consider increasing tissue thickness and using the appropriately sized Tile-slide Spacer.   |
|                         | Over digestion or over lysis of sample                   | Likely to occur from excessive pestle grinding. Reduce the amount and duration of pestle grinding being performed.  |
|                         | Insufficient dissociation                                | Depending on the tissue composition, prolonged dissociation time may be required to sufficiently break up the tissue and lyse the cells. It is possible that adding pestle grinding may be required to mechanically dissociate the tissue.  |
|                         | Excessive dissociation                                   | Excessive or aggressive dissociation may lead to over lysis of the cells and lysis of the nuclei. Reduce the dissociation time accordingly to optimize the yield of intact nuclei.  |
|                         | Inaccurate nuclei counting                               | For manual counting, ensure a clean preparation, establish clear counting rules, verify optimal dilutions, and perform multiple counts. Alternatively, switch to automated cell counting.   |
|                         | Insufficient deparaffinization                           | The presence of paraffin wax inhibits the dissociation of the tissue. Insufficient deparaffinization can be determined by inconsistent or patchy H&E staining. If necessary, incorporate an additional Histo-Clear step and/or extending the Histo-Clear incubation to 5–10 min.  |

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|  | Insufficient tissue collected within section                          | Depending on the FFPE tissue block, the user is either too shallow or too deep in the block. If too shallow, face the block until the desired region is reached. For thinner tissues and samples, such as TMAs and core needle biopsies, use a thinner sectioning setting to minimize excessive tissue loss. If the block is too deep, the tissue sample is close to depletion and consider replacing the block.        |
| <b>Excessive nuclei clumping</b>                                   | High amount of ambient genomic DNA                                    | Excessive lysis of nuclei, leading to the release of genomic DNA. Reduce dissociation time and trituration to minimize nuclei lysis.  |
|  | Residual membrane leading to stickier nuclei                          | Increase BSA concentration within the Resuspension Buffer and pass through 20 to 40 µm pore filter depending on sample.   |
| <b>Misalignment of fixture or failure to tighten</b>               | Cross-threading of Tightening Ring and Bottom Plate                   | Cross-threading happens when the components are assembled at an angle. <b>DO NOT</b> forcibly tighten when cross-threading occurs. Disassemble the fixture and reset the positioning of the Tightening Ring. If severely jammed, spray thread with silicone lubricant, wait ~15 min, and try to loosen.   |
|  | Damage to the threading of either the Tightening Ring or Bottom Plate | <b>DO NOT</b> use it in experiments. Contact Technical Support ( <a href="mailto:Technical_Support@takarabio.com">Technical_Support@takarabio.com</a> ) for more information.   |
| <b>Beads coming off the tile during fixture disassembly</b>        | The Trekker FX was scratched during handling and assembly             | Proceed if beads are <5% of the final single-nuclei suspension. If beads are >5% of the final suspension, it is not recommended to proceed with the sample as significant bead contamination would cause clogging during single-nucleus capture and increase background noise. Contact technical support ( <a href="mailto:Technical_Support@takarabio.com">Technical_Support@takarabio.com</a> ) for more information. |
| <b>Trekker final library missing expected peak</b>                 | UV cleavage unsuccessful  | Ensure UV lamp settings are correct, and the UV lamp is functioning. A visual check can be performed by shining the light on a Kimwipe or white paper to see it glow. <b>DO NOT</b> look directly at the bulb when on. If no light is observed, contact technical support ( <a href="mailto:Technical_Support@takarabio.com">Technical_Support@takarabio.com</a> ) for more information.                                |
|  | Incorrect primers were used in library prep                           | Ensure correct primers and cycle numbers are used. For the Pre-Amplification PCR (Step 4.2 for Flex v1, Step 5.2 for Flex v2), ensure that Amp Mix C (PN 2001311) and Pre-Amp Primers C (PN 2000953) are used in the reaction. For the Sample Index PCR (Step 6.1 for Flex v1, Step 7.1 for Flex v2), ensure that Amp Mix (PN 2000047) and Dual Index Plate TN Set A (PN 3000510) are used in the reaction.             |
| <b>Trekker final library contains significant off target peaks</b> | Over amplification  | Ensure the correct input amount and cycle number (different from 10x user guide) was used for index PCR.  |
| <b>Low percentage of spatially tagged nuclei</b>                   | Low sequencing depth  | Trekker spatial tagging efficiency may differ between tissue types. Increasing sequencing depth to 5,000 reads per nuclei from the recommended 2,000 reads per nuclei may be required.  |
|  | Wrong tile-slide spacer   | Use of the thicker Tile-slide Spacer A on sections <16 µm will result in a reduction in tissue spatial tagging due to the   |

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|   |  | larger resulting gap distance between the tissue and the tile. Use the Tile-slide Spacer B for sections between 10–15 µm thickness.   |
|   | Wrong tile used in experiment or input in the pipeline | Double check the ID of the Trekker FX 10x10 Tile-slide used in the experiment. If ID is not available or problem persists, contact Technical Support ( <a href="mailto:Technical_Support@takarabio.com">Technical_Support@takarabio.com</a> ).  |
|   | Excessive inclusion of non-spatially tagged tissue     | Tissue was not trimmed prior to tissue-tile alignment and Fixture assembly. Using tissues larger than the 10x10 mm size of the Trekker FX Tile would result in relative increase in non-spatially tagged tissue and nuclei. This translates to a decrease in spatially tagged nuclei in the single-cell preparation. Prior to deparaffinization, trim tissue with a clean single-edge razor blade to fit within the 10x10 mm tile (see <a href="#">Figure 13</a> ). |
|   | Insufficient spatial tagging                           | Ensure UV lamp settings are correct, and the UV lamp is functioning. Ensure that there is no air bubble present during spatial tagging.   |
| <b>Beads coming off of the tile during dissociation</b> | The tile was scratched during the dissociation         | Proceed if beads are <5% of the final single-nuclei suspension. If beads are >5% of the final suspension, it is not recommended to proceed with the sample as significant bead contamination would cause clogging during single-nucleus capture and increase background noise. Contact Technical Support ( <a href="mailto:Technical_Support@takarabio.com">Technical_Support@takarabio.com</a> ) for more information.   |

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