DEMONSTRATED PROTOCOL

Visium Spatial Protocols – Tissue Preparation Guide

Overview

The Visium Spatial Gene Expression Solution measures the total mRNA in tissue sections and requires a Visium Spatial slide with intact tissue sections as input. Proper tissue handling and preparation techniques preserve the morphological quality of the tissue sections and the integrity of mRNA transcripts. This is critical for downstream library preparation and generation of high quality sequencing data using the Visium Spatial Gene Expression protocols.

The Tissue Preparation Guide provides guidance on:

- Selecting appropriate Visium Spatial slides specific to the Visium Spatial protocol being used.
- Best practices for handling tissue samples and Visium Spatial slides before and after cryosectioning.
- Freezing and embedding tissue samples prior to cryosectioning.
- Cryosectioning of tissue samples and placement of sections on Visium Spatial slides.

Additional Guidance

This protocol was demonstrated using mouse brain tissues. However, the general principles for tissue preparation, cryosectioning, and storage are expected to be compatible with many tissue types (visit the 10x Genomics support website for a detailed list). Additional optimization may be required for the preparation of specialized tissues, such as tissue with high fat content. Refer to the 10x Genomics Support website for additional resources, including How-to-Videos.

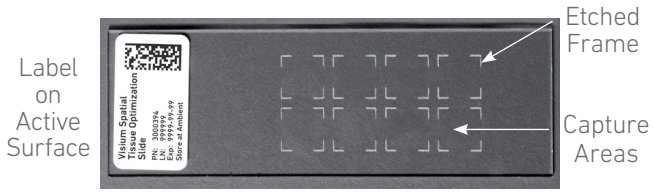
The slides prepared using the Tissue Preparation Guide can be used with:

- Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238)
- Visium Spatial Gene Expression Reagent Kits User Guide (CG000239)

Visium Slide Selection

Visium Spatial Tissue Optimization Slide (PN-3000394)

- Used with Visium Spatial Gene Expression Reagent Kits – Tissue Optimization User Guide (CG000238) to identify optimum permeabilization time for a specific tissue type.
- Includes 8 Capture Areas, each covered with oligonucleotides for mRNA capture.
- Each Capture Area is 8 x 8 mm and is surrounded by an etched frame.
- A readable label defines the active surface of the slide.



Visium Spatial Gene Expression Slide (PN-2000233)

- Used with Visium Spatial Gene Expression Reagent Kits User Guide (CG000239) to generate Visium Spatial Gene Expression libraries.
- Includes 4 Capture Areas, each with ~5,000 unique gene expression spots.
- Each Capture Area is 6.5 x 6.5 mm and is surrounded by a fiducial frame for a total area of 8 x 8 mm.
- A readable label with a serial number defines the active surface of the slide.

Tips & Best Practices

Best Practices & Icons

- Best practices for handling any tissues include using sterile techniques, nuclease-free reagents and consumables.

Tips & Best Practices section includes additional guidance

Signifies critical step requiring accurate execution

Troubleshooting section includes additional guidance

Cryosectioning Temperature

- Cryosectioning temperatures impact tissue section integrity. A temperature setting of **–20°C** for blade and **–10°C** for the specimen head is recommended.
- The temperature settings depend upon the local conditions, tissue types, and the cryostat used and should be optimized based on the quality of resulting tissue sections.
- During prolonged sectioning periods, allow the cryostat temperature to equilibrate by briefly closing the chamber.

Tissue Scoring

- A tissue section of ≤6.5 x 6.5 mm is compatible with Visium Spatial slides.
- OCT block with embedded tissue can be trimmed with a razor blade to fit the Capture Areas.
- Large tissue samples can be scored during sectioning to generate smaller samples to fit the Capture Areas.
- Scoring can be done by making a shallow incision (~1 mm deep) on the cutting surface of the tissue with a razor blade.
- The incision should be shallow. A deep incision may lead to tissue damage and disintegration.
- Once a tissue has been scored, use extra care during sectioning and section handling.

Sectioning Speed

- Sectioning speed depends upon the desired thickness of the sections and the condition of the tissue. Harder and thicker sections require slow sectioning speed.
- Faster sectioning speed may lead to cracks or tears in the sections or damage to the tissue block or cryostat.

Section Thickness

- Recommended section thickness for most tissue types is 10 µm. Tissues with higher fat content (e.g., breast tissue) may require thicker sections.
- Visit the 10x Genomics support website for guidance on section thickness for compatible tissue types.

Handling Visium Slides

Handling Visium Spatial Slides Before Sectioning:

- Store unused slides in original packaging and keep sealed. DO NOT remove the desiccant.
- Equilibrate slides to cryostat temperature before proceeding with cryosectioning to prevent quick melting of the sample and the associated RNA degradation.

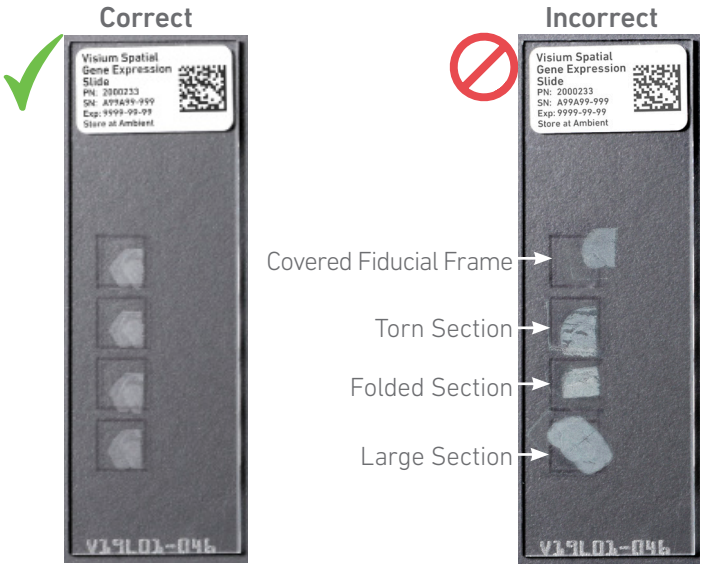
Handling Visium Spatial Slides Containing Tissue Sections:

- Maintain slides containing tissue sections in a low moisture environment.
- Keep slides cold and transport on dry ice.
- DO NOT leave slides at **room temperature**, especially with fresh sections as the resulting condensation will cause tissue disintegration.
- Store slides in a sealed container. If necessary, place slides in a secondary container, such as a resealable bag.
- Store slides individually (one slide per container) for up to **4 weeks** at **–80°C** to avoid multiple freeze-thaw cycles.

Section Placement on Slides

- Place the tissue section within the fiducial frame or the etched frames of the Capture Area on the pre-equilibrated Visium Spatial slides. Avoid covering the frames of the Capture Areas with the tissue.
- The section on the slides should be uniform without any cracks, tears, or folds.
- Only one section should be placed within each Capture Area.

Visium Spatial Gene Expression Slide



- For Visium Spatial Tissue Optimization Slide, place tissue sections on 7 of the 8 Capture Areas. Leave one Capture Area empty for positive RNA control.

Visium Spatial Tissue Optimization Slide

Practice Section Placement

- Create representative frames on a 75 x 25 x 1 mm plain glass slide using the Visium Spatial Slide Layout (see Appendix).
- Frames should be drawn on the back of the slide.
- Practice correct section placement within the representative frames.

1. Tissue Freezing & Embedding

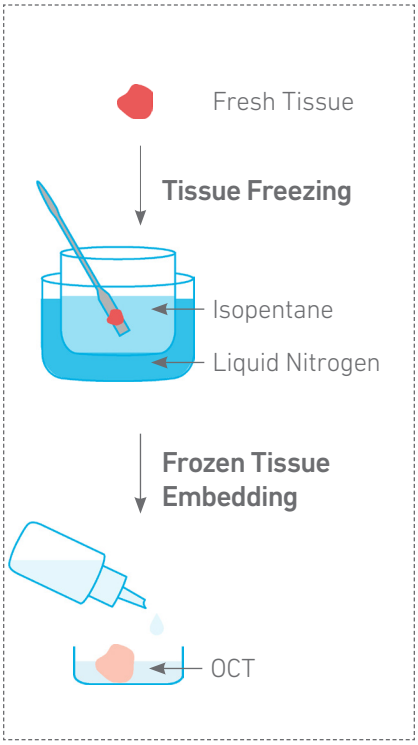
1.0 Overview

This chapter provides guidance on tissue freezing and embedding. Freshly obtained tissue samples must be snap frozen to prevent RNA degradation and avoid crystal formation, which can lead to morphological damage to the tissue. Once frozen, tissue samples are embedded in a freezing and embedding compound, Optimal Cutting Temperature (OCT), to preserve the structure of the tissue and to provide structural support during cryosectioning.

Alternatively, perform simultaneous freezing and embedding in OCT for tissues with crevices/gaps or tissues that have a tendency to curl (see Appendix for details).

Tissue Freezing

A bath of isopentane and liquid nitrogen is used to freeze the freshly obtained tissue. Tissue should not be placed directly in liquid nitrogen as the temperature difference may cause boiling on the surface of the tissues, leading to air pockets and uneven freezing. This may crack and morphologically damage the tissue.



Frozen Tissue Embedding

Prior to cryosectioning, frozen tissue samples are embedded in OCT. Unlike paraffin or resin-based embedding techniques, OCT does not interact with proteins and other molecules that impact antigenicity.

OCT embedding of the tissue offers the following advantages:

- Preserves the structure of the tissue and provides structural support during cryosectioning.
- Maintains an optimal temperature during sectioning, thus leading to smooth sections.
- Compatible with multiple staining procedures due to its water solubility.

1.1 Reagents & Consumables

Tissue Freezing		
Vendor	**Item**	**Part Number**
Millipore Sigma	Isopentane (2-Methylbutane)	270342
VWR	Stainless Steel Beaker (250 ml)	89075-592
	Specimen Forceps, Straight, 203 mm (8")	82027-436
	Specimen Forceps, Straight, 152 mm (6")	82027-438
	Round/Tapered Spatula, Stainless Steel	82027-490
Wheaton	WHEATON 5 ml CryoELITE Tissue Vial	W985100

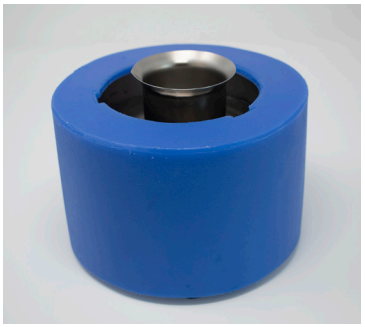
Frozen Tissue Embedding		
Vendor	**Item**	**Part Number**
VWR	TissueTek O.C.T. Compound	25608-930
	Disposable Based Molds (15 x 15 mm) Dependent on the tissue size	60872-488

Additional Materials		
-	Dry Ice	-
-	Liquid Nitrogen	-
-	Razor Blades	-
-	Dewar for Liquid Nitrogen Choose appropriate size based on the size of the steel beaker used	-

1.2 Tissue Freezing

Items	Preparation & Handling
Prepare	
☐ **Isopentane and liquid nitrogen bath**	Fill two-thirds of a metal beaker with isopentane (sufficient to fully submerge the tissue) and place in a liquid nitrogen dewar (same level as isopentane) to allow sufficient contact. Incubate **15 min**. Isopentane will slowly freeze if left too long in the liquid nitrogen bath. When this occurs, briefly remove the metal beaker containing isopentane from the liquid nitrogen bath. Once thawed, place the beaker back in the liquid nitrogen bath.
☐ **Tissue**	Using a rolled up laboratory wipe, absorb excess blood or solution from the surface of the tissue to limit ice crystal formation.
☐ **Pre-cooled Cryovial**	Pre-cool a WHEATON CryoELITE cryovial on dry ice.

Isopentane and Liquid Nitrogen Bath



a. Using either forceps or a spatula, lower the tissue into the isopentane until fully submerged. Keep tissue submerged for ~**1 min** or until frozen. The freezing time may vary based upon the tissue type and size.

b. Once frozen, transfer the tissue to the pre-cooled WHEATON CryoELITE cryovial on dry ice.

STOP

c. Store frozen tissue at **–80°C** for **long-term** storage or **immediately** proceed to the next step (Frozen Tissue Embedding).

To prevent evaporation and dehydration of the tissue sample, snap-frozen tissue sample must be stored in a sealed container.

1.3 Frozen Tissue Embedding

Items	Preparation & Handling
Prepare	
☐ **Powdered dry ice**	Use a mortar and pestle to prepare powdered dry ice.
☐ **Chilled OCT**	Place OCT in ice for ≥**30 min**.
☐ **Pre-cooled forceps**	Place forceps in dry ice for ≥**30 min**.
Confirm	
☐ **Cryomold**	The cryomold used for embedding should be of appropriate size to fit the tissue sample.

a. Label an appropriately sized cryomold to mark the orientation of the tissue.
Label the cryomold before adding OCT and tissue. The OCT will quickly turn white once frozen, making it hard to determine tissue orientation later.

b. Fill the cryomold with chilled OCT without introducing bubbles.

c. Remove frozen tissue from **–80°C** and transfer in dry ice.

d. Using pre-cooled forceps, place the frozen tissue into the OCT, covering any exposed surfaces with additional OCT.
Confirm there are no bubbles, especially near the tissue.

e. **Immediately** place the cryomold containing tissue and OCT on powdered dry ice.

f. Wait until the OCT is completely frozen.

STOP

g. Store the OCT embedded tissue block in a sealed container at **–80°C** for **long-term** storage or **immediately** proceed to Cryosectioning & Section Placement.

A WHEATON CryoELITE cryovial or a resealable bag can be used for storing the tissue block. Remove the tissue block from the cryomold and trim it using a razor blade to fit into the cryovial.

Failure to use a sealed container for storage may dehydrate and damage the tissue.

Frozen Tissue Embedding

Tissue in OCT

After Embedding

OCT Tissue Block Trimming

Before Trimming

After Trimming

2. Cryosectioning & Section Placement

2.0 Overview

This chapter provides guidance on cryosectioning of the OCT embedded tissue and placement of the tissue sections on the Visium Spatial slides.
Choose appropriate slide based on the Visium Spatial protocol being used.

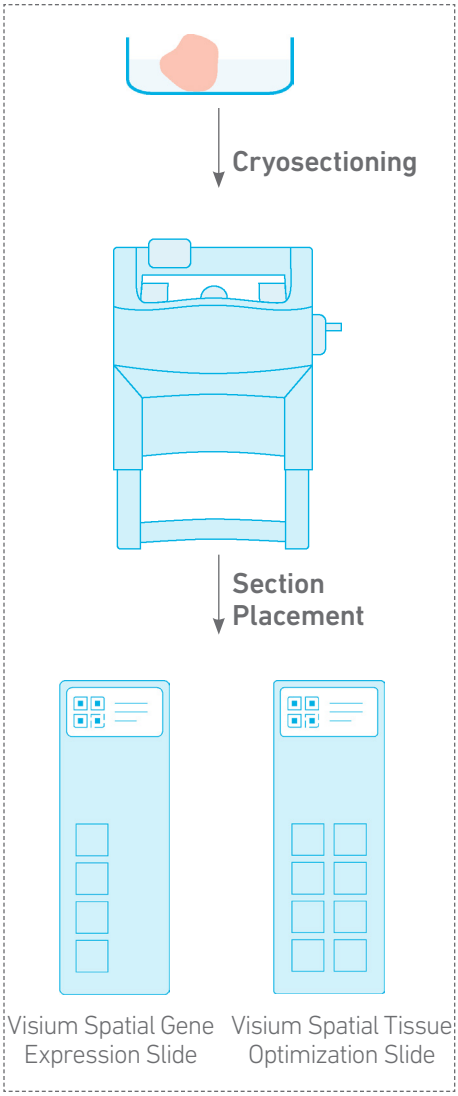
Cryosectioning

OCT embedded tissue blocks are removed from the **–80°C** storage and cryosectioned in a cryostat to generate appropriately sized sections for Visium Spatial slides while keeping the samples frozen.

Section Placement

Tissue sections are placed within the frames of Capture Areas on Visium Spatial slides. Only one section should be placed within each Capture Area.

For Visium Spatial Tissue Optimization Slide, 7 of the 8 Capture Areas are used for tissue and one is left empty for a positive RNA control. Only one tissue type and section thickness should be tested per slide.



2.1 Reagents & Consumables

Vendor	Item	Part Number
VWR	TissueTek O.C.T. Compound	25608-930
	Sterile Centrifuge Tubes with Flat Caps, 50 ml	82018-050
10x Genomics	Visium Spatial Tissue Optimization Slide/ Visium Spatial Gene Expression Slide	3000394/ 2000233
Thermo Fisher Scientific	CryoStar NX70 Cryostat Vacutome, Low Profile Blade Carrier	957020
	Shandon ColorFrost Plus Slides 75 x 25 x 1 mm (Optional)	6776214
	Flat cryostat brush, 10 mm	334160
	Brush, small beveled	334171
	Magnetic Brush, big	334172
Fisher Scientific	Thermo Scientific CryoStar NX70 Specimen Chuck	14-071-413
	Simport Scientific LockMailer Tamper Evident Slide Mailer (Alternatively, use a 50-ml centrifuge tube)	22-038-399
	MX35 Ultra Microtome Blade Low Profile	30-538-35350
	Glass Anti-Roll Plate	A78930200
Additional Materials		
-	Razor Blades	-
-	Dry Ice	-
-	Tissue Forceps	-

Cryostat Chamber Specifications

This protocol describes the use of a Cryostar NX70 Cryostat with specific capabilities. Alternatively, use a different cryostat with following features.

Function	Notes
Main Cryochamber	Maintains stable temperatures from –10°C to –20°C
Cryostat Blade	Separate and adjustable temperature control Maintains stable temperatures from –35°C to –5°C
Specimen Head	Separate and adjustable temperature control Maintains stable temperatures from –50°C to +10°C X-axis and Y-axis adjustment
Blade Holder Base	Adjustable cutting angle Adjustable blade position Section thickness 10-50 µM
Cryobar	Rapid cooling

2.2 Cryosectioning

Items	Preparation & Handling
Adjust	
☐ **Cryostat temperature settings**	Turn cryostat on to pre-cool chamber. Recommended sectioning temperature is **–20°C** for cryostat blade and **–10°C** for the specimen head. Follow manufacturer's manual for detailed operations.
Equilibrate	
☐ **Visium Spatial slides to the cryostat chamber temperature**	Slides should be cooled down to cryostat temperature for **≥30 min**. Warm slides will lead to quick melting of the sections and degradation of RNA.
☐ **OCT embedded tissue block to cryostat chamber temperature**	OCT embedded tissue block stored at **–80°C** must be equilibrated to cryostat chamber temperature for **30 min** before sectioning. If the tissue block is too cold, it will lead to section cracking. If the tissue block is too warm, it will lead to section compression or crumpling.

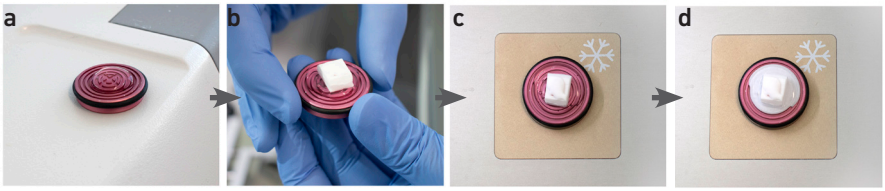
Mount OCT Embedded Tissue Block on the Specimen Stage:

a. Fill the specimen stage (chuck) with OCT.

b. Place the OCT embedded tissue block on the stage with the cutting surface facing away from the stage

c. Place the stage and the tissue block on the cryobar inside the cryostat chamber.

d. Allow the OCT and the tissue block to freeze and adhere to the specimen stage.



Remove Excess OCT by Cryosectioning:

a. Once frozen, install the stage with the tissue block on to the specimen head of the cryostat and start sectioning to remove excess OCT.

b. Sectioning conditions vary across different tissues and cryostats. Follow manufacturer's recommendation for cryosectioning.

c. Continue sectioning until the tissue is visible.

RNA Quality Assessment:

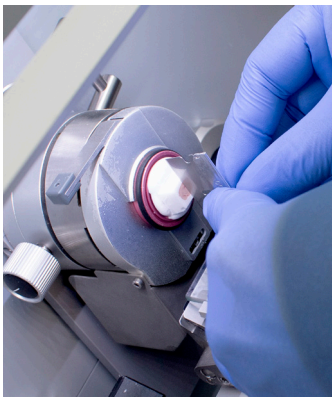
It is recommended to assess RNA quality of the tissue block at this stage by calculating RNA Integrity Number (RIN) of freshly collected tissue sections. See Appendix for details. RIN should be ≥ 7 and RNA quality assessment should be done before placing the tissue sections on Visium Spatial slides. Various factors could lead to low RIN scores, such as specific tissue types, diseased or necrotic tissues, sample preparation and handling.

Tissue Scoring:

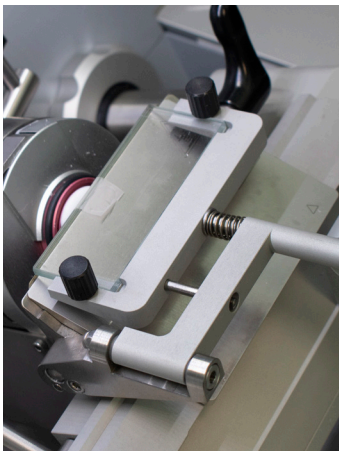
Large tissue samples can be scored during sectioning to generate smaller samples to fit the Capture Areas. To score, make a shallow incision (~1 mm deep) on the cutting surface of the tissue with a pre-cooled razor blade. The incision should be shallow. A deep incision may lead to tissue damage and disintegration. Once the tissue has been scored, extra care must be taken during sectioning and section handling.

Example: To examine a specific region within one hemisphere of the mouse brain, scoring can be done by making a ~1 mm shallow incision at the midline of the brain.

Tissue Scoring



2.3 Section Placement

Items	Preparation & Handling
Confirm	
☐ **Section thickness setting**	Recommended section thickness is 10 µm for most tissue types. Visit the 10x Genomics support website for guidance on section thickness for compatible tissue types.
☐ **Anti-roll plate is in place**	Anti-roll plate prevents rolling of tissue sections. Optimize the position of anti-roll plate based on the tissue block size. If possible, adjust the position of anti-roll plate before reaching area of interest. Position of Anti-roll Plate 
☐ **Specimen head temperature**	Confirm the temperature of the specimen head. If the sections appear cracked, the specimen head is too cold. If the sections appear crumpled, the specimen head is too warm. Adjust temperature accordingly.
Practice	
☐ **Section placement on a plain glass slide**	Create representative frames on a 75 x 25 x 1 mm plain glass slide and practice section placement within the frames before working with the Visium Spatial slides. See Appendix for the Visium Spatial Slide Layout.

2.3 Section Placement

a. Practice section placement on plain glass slides. See Appendix for Visium Spatial slide layout.

b. Once desired tissue section is obtained, carefully flatten it out by gently touching the surrounding OCT with cryostat brushes.

c. Place the section within a Capture Area on the pre-equilibrated Visium Spatial slide by gently touching the section with the active surface of the slide.

DO NOT place sections on a room temperature slide. Slide should be equilibrated to cryostat chamber. Avoid contact between the active surface of the slide and the cryostat as it can damage the oligonucleotides and decrease the capture efficiency of the Visium Spatial slides.

d. **Immediately** place a finger on the backside of the Capture Area on the slide for a few seconds to allow the section to adhere to the slide.

Ensure that the entire tissue section is fully adhered to the slide and the slide is inside the cryostat chamber throughout section placement.
DO NOT remove the slide from the cryostat chamber at any point during sectioning and tissue placement.

Immediately place the slide with tissue section on the cryobar to freeze the section. Continue transferring sections on the remaining Capture Areas.

For Visium Spatial Tissue Optimization Slide, place sections on 7 of the 8 Capture Areas, leaving one Capture Area empty for positive RNA control. Ensure that serial sections from the same tissue block are placed on the Visium Spatial Tissue Optimization slide.

e. Transfer the slide containing tissue sections to a slide mailer placed in dry ice.

f. Store slides at **–80°C** for up to **4 weeks** or **immediately** proceed to Visium Spatial protocols.

Store slides individually (one slide per container) in a sealed container. If necessary, place the slides in a secondary container, such as a resealable bag.

Maintain slides containing sections in a cold and low moisture environment.
DO NOT expose slides to **room temperature** as the resulting condensation will cause tissue disintegration.

See Tips and Best Practices for handling slides.

Shipping of Slides:

If needed, slides containing tissue sections can be shipped on dry ice. See Appendix for detailed Shipping Guidelines.

Leftover Tissue Block Storage:

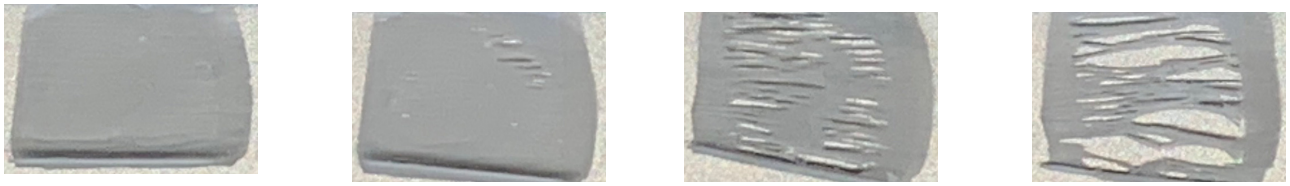
- Remove leftover tissue block attached to the specimen stage from the cryostat's specimen head and place onto cryobar.
- Cover the exposed tissue with a thin layer of OCT and allow to freeze.
- To separate the frozen tissue block from the stage, lift the tissue block and the stage from the cryobar and lightly warm the stage with hands or an aluminum block at room temperature

  DO NOT let the block and tissue to fully melt, as this will severely damage the tissue. Separation of the tissue block from the specimen stage is optional. The frozen tissue block can be stored attached to the specimen stage in a sealed container at –80°C.
- **Immediately** place the tissue block in dry ice. Ensure that the melted areas have refrozen.
- Store in a sealed container at **–80°C** for **long-term** storage.

Troubleshooting

Impact of Cryostat Specimen Head Temperatures on Tissue Tearing

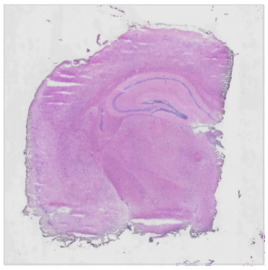
-10°C | -14°C | -20°C | -30°C



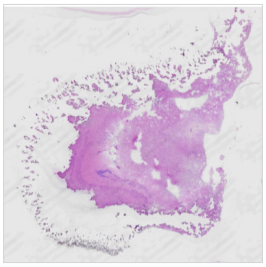
Normal Section

Torn sections. Adjust and confirm specimen head temperatures.

Impact of Condensation on Tissue Sections

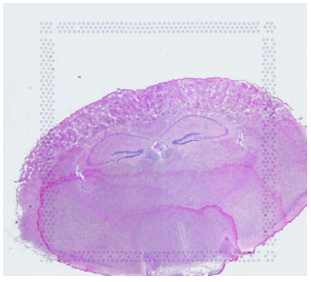


No Condensation. Intact tissue section.

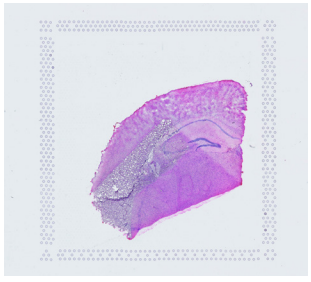


Tissue degraded due to condensation. DO NOT leave slides at room temperature, especially with fresh sections as the resulting condensation will cause tissue disintegration.

Incorrect Placement of Tissue Sections



Fiducial frames covered



Folded tissue section

Overlapping sections

Practice correct section placement on blank glass slides before proceeding with Visium Spatial slides. See Appendix for Visium Spatial slide layout.

Images shown are tissue sections fixed with methanol and stained with hematoxylin and eosin (H&E).

Appendix

Simultaneous Freezing & Embedding

Items	Preparation & Handling
Prepare	
☐ **Isopentane and liquid nitrogen bath**	Fill two-thirds of a metal beaker with isopentane (sufficient to fully submerge the tissue) and place in a dewar of liquid nitrogen (same level as isopentane) to allow sufficient contact. Incubate for **15 min**.
☐ **Tissue**	Using a rolled up laboratory wipe, absorb excess blood or solution from the surface of the tissue to limit ice crystal formation.
Confirm	
☐ **Cryomold**	The cryomold used for embedding should be of appropriate size to fit the tissue sample.

See Table 1.1 Reagents & Consumables for a detailed reagents list.

a. In a petri dish, carefully and thoroughly coat fresh tissue sample with **room temperature** OCT. Confirm there are no bubbles on the surface of the tissue.

b. Using a spatula, place the OCT–coated tissue into an appropriately sized cryomold. Label the cryomold to mark the orientation of the tissue.

c. Fill the cryomold with additional OCT, ensuring that the tissue is completely covered. Confirm there are no bubbles, especially near the tissue.

d. Using forceps, lower the cryomold containing embedded tissue into the isopentane without fully submerging. Keep cryomold in contact with isopentane until the OCT has solidified and turned white.

If isopentane and liquid nitrogen are not available, powdered dry ice or a metal block chilled in dry ice can be used as an alternative.

e. Once frozen, place the cryomold on dry ice.

STOP

f. Store frozen embedded tissue in a sealed container at **–80°C** or liquid nitrogen for **long-term** storage or **immediately** proceed to Cryosectioning and Section Placement.

Failure to use a sealed container for storage may dehydrate and damage the tissue.

RNA Quality Assessment

This section provides guidance on assessing the quality of the OCT embedded tissue blocks by calculating its RNA Integrity Number (RIN).

Vendor	Item	Part Number
Qiagen	RNeasy Mini Kit (50)	74104
	QIAshredder (50)	79654
Thermo Fisher Scientific	RNaseZap RNase Decontamination Solution	AM9780
	Nuclease-free Water	AM9937
Millipore Sigma	2-Mercaptoethanol	M6250-100ML
Eppendorf	DNA LoBind Tubes, 1.5 ml	022431021
Agilent	Agilent RNA 6000 Pico Kit	5067-1513
	Agilent RNA 6000 Nano Kit	5067-1511
Additional Materials		
-	Dry Ice	-
-	Tissue Forceps	-
-	Razor Blades	-

Pre-cool microcentrifuge tubes, cooling block, and forceps in cryostat chamber or at **–20°C** to prevent premature melting of the tissue sections.

a. Prepare 10 sections, each at 10 µm thickness.

b. Using the cooled forceps, pick up the sections and place inside a pre-cooled microcentrifuge tube.

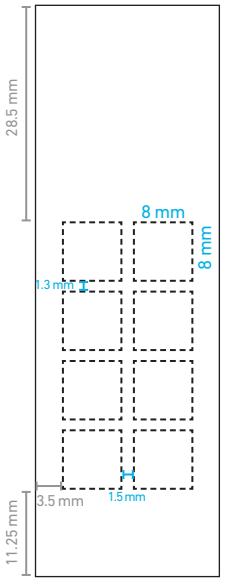
c. Proceed to RNA isolation using Qiagen RNeasy Mini Kit or store at **–80°C** for **long-term** storage. Follow manufacturer's recommendation for RNA isolation. The section on Purification of Total RNA from Animal Tissues can be used for RNA isolation.

d. Store purified RNA at **–80°C** for **long-term** storage or **immediately** proceed to RIN calculation using either Agilent RNA 6000 Nano or Pico Kit. Follow manufacturer's instructions (Agilent) for RIN calculation.
The Visium Spatial protocol was optimized using samples with RIN ≥7.

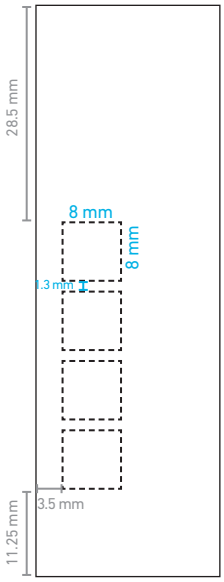
Visium Spatial Slide Layout

A layout of Capture Areas of Visium Spatial slides is shown below and can be used to create representative frames on plain glass slides with dimensions (75 x 25 x 1 mm) similar to Visium slides to practice tissue section placement. The frames should be drawn on the back of the slide.

Capture Areas – Tissue Optimization Slide



Capture Areas – Gene Expression Slide



The slide dimensions represent a 75 x 25 x 1 mm laboratory glass slide; printer settings may impact the image scaling.

For Visium Spatial Tissue Optimization Slide, each Capture Area is 8 x 8 mm and is surrounded by an etched frame. For Gene Expression Slide, each Capture Area is 6.5 x 6.5 mm and is surrounded by a fiducial frame for a total area of 8 x 8 mm.

Shipping Guidelines

- Place slides in a slide mailer. If multiple slides are being shipped, ensure that there is sufficient space in between the slides to avoid contact.
- Add folded paper towel to prevent excessive movement of the slides during shipping.
- Place the mailer in a tightly sealed secondary container to limit exposure.
- Samples can be shipped overnight in dry ice, provided there is enough dry ice to account for transit and delivery times.
- Refer to the local institution or delivery service for detailed instructions on shipping samples in dry ice.

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