	Bent-tip tweezers (Highly recommended)	**Fine Science Tools**	**11251-33**	Tissue Prep, Conjugation, Staining Tissue, CODEX™ Experiment
	6-Well TC Plates - Does not need to be tissue cultured treated.	VWR	10861-554	Staining Tissue
	1mL, 1.5 mL, 2 mL tubes	Customer choice	---	Staining Tissue, CODEX™ Experiment
	Amber 1.5 mL tubes	Customer choice	---	Reporter Prep, CODEX™ Experiment
	Serological Pipet	Customer choice	---	Tissue Prep, Staining Tissue, CODEX™ Experiment
	5, 15, 50 ml conical tubes	Customer choice	---	Staining Tissue
Biologics/reagents	16% Paraformaldehyde			Staining Tissue
	1X PBS	Life Technologies	14190144	Conjugation, Staining Tissue
	Poly-L-Lysine 0.1%	Sigma-Aldrich	P8920	Tissue Prep
	Drierite Absorbents	Fisher Scientific	23-116582	Conjugation, Staining Tissue
	Nuclease-Free Water	Thermo Fisher Scientific	AM9938	Reporter Prep
	Fluoromount-G™ (optional)	Thermo Fisher Scientific	00-4958-02	Screening
	ddH_2O or MilliQ H_2O	Customer choice	---	CODEX™ Experiment
Solvents	Acetone	Sigma-Aldrich	650501-1L	Staining Tissue
	Methanol	Sigma-Aldrich	34860-1L-R	Staining Tissue
	DMSO - ACS reagent, ≥99.9%	Sigma-Aldrich	472301-4L	Staining Tissue, CODEX™ Experiment
Instrumentation	UPS	APC Back-UPS Pro 1500	BR1500G	CODEX™ Experiment

Coverslips Electron Microscopy Sciences 72204-01 Tissue Prep

Chapter 3. Coverslip and Tissue processing

This section of the user manual describes the required procedures of tissue preparation and storage for CODEX® experiments and must be completed before proceeding further with the experimental workflow. Fresh frozen or FFPE tissues are sectioned directly onto poly-lysine-coated coverslips. Using microscope slides, uncoated coverslips, and tissue preparations deviating from this protocol may not be compatible with CODEX® platform.

Note *In Fluorescence imaging experiments it is important to select tissues with low autofluorescence. Guidelines on how to investigate the level of autofluorescence are to be found in Appendix E*

3.1 Poly-lysine Coverslip Preparation

This section describes the process of creating Poly-lysine-coated coverslips that are used for the tissue slices in the CODEX® experiment workflow. Tissue slices for CODEX® can only be placed on provided 22 mm × 22 mm coverslips after proper poly-lysine preparation.

Incubating Coverslips

Guidelines

Workflow Timing

- The preparation of Poly-lysine coated coverslips has a minimum incubation of 12 hours. It is recommended that coverslips are treated at least 1 day before tissue sectioning.
- The coverslips can incubate in poly-lysine for a maximum of 1 week.
- Poly- lysine coated coverslips must be used within 2 months.

Pre-Experiment Preparation

Materials included in kit:

Contents	Kit	Storage
Coverslips	à la carte	15°C to 30°C

Materials NOT included in kits:

- 0.1% Poly-lysine solution
- Glass beaker (0.5L)
- Rubber band
- Plastic wrap

Incubating Coverslips

a. Remove the coverslips from box.
b. Gently place coverslips at the bottom of the glass beaker.
c. Slowly swirl the beaker to spread the stacks of coverslips.
d. Add *ca*. 7 mL of poly-lysine solution above the coverslips to ensure that all coverslips are fully covered.
e. Slowly swirl the solution by rotating the beaker at a 45° angle for 1 minute, ensuring that the entire surface of all coverslips is fully immersed in solution

Note *Coverslips should be dispersed to maximize the surface area of each coverslip exposed to the solution. Minimize the number of coverslips sticking and overlapping with one another.*

f. Cover the beaker with plastic wrap and seal with a rubber band to prevent evaporation.
g. Leave coverslips in poly-lysine solution for a minimum of 12 hours and up to one week at room temperature.

INCUBATE Minimum 12-hour incubation

STOPPING POINT

- Leave coverslips in Poly-lysine solution for a minimum of 12 hours and up to one week.

Washing and Storing Coverslips

Guidelines

Coverslips

- After incubation, coverslips can be stored for up to 2 months at room temperature.
- To prevent removal of poly-lysine, do not soak in water for >1 minute during each washing step.

Reagents

- Milli-Q ultrapure water (Type 1) can be used in place of ddH_2O. DIH_2O is not recommended.

Pre-Experiment Preparation

Materials NOT included in kits:

- Petri dish or similar container
- Paper towels
- ddH_2O

Washing Coverslips

a. Gradually pour poly-lysine solution into the proper waste container.
b. Fill the beaker containing the coverslips to half volume with ddH_2O.
c. Swirl the contents to mix the solution.
d. Let the beaker and coverslips sit for 1 minute.
e. Slowly pour off the water into the sink.
f. Repeat steps b-e 6 more times for a total of 7 washes.
g. Fill the beaker containing coverslips to half volume with ddH_2O.
h. Place two sets of paper towels on the bench top.
i. Remove the coverslips from the water, placing them on top of the first set of paper towels. Ensure coverslips are not overlapping to allow proper drying.

Note *Coverslips can be removed from the beaker in batches.*

j. Invert each coverslip. Dry the reverse side on the second set of paper towels.
k. Leave the coverslips on the paper towels to dry.
l. When they are dry, the Poly-lysine-coated coverslips can be stored in a petri dish or similar container.

STOPPING POINT

- Place all Poly-lysine coated coverslips in a petri dish for storage for up to 2 months.

3.2 Tissue Storage

A storage box can easily be created using a cardboard freezer box with tube inserts.

Guidelines

Storage Box

- It is best to avoid boxes with holes in the bottom because they can dry the tissues out over time.
- Prepare the box ahead of use.
- To ensure box is at optimal temperature prior to tissue storage, place box in cryostat chamber while slicing tissue.

Pre-Experiment Preparation

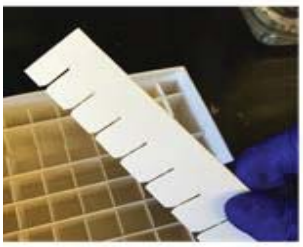
Materials NOT included in kits:

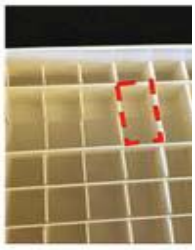
- Cardboard freezer box with tube inserts: 5 × 5 × 2 in (12.7 × 12.7 × 5 cm), USA Scientific 9023-4981
- Lab Tape

Storage Box Creation

a. Start with a standard cardboard freezer box with tube inserts.

b. Create slots measuring 26 mm × 14 mm by removing every other insert in one direction.





c. Tape the inserts to the sides of the cardboard box.

CRITICAL To prevent tissue slices from slipping below the inserts and stacking on top of one another, it is critical to tape the inserts in a manner to ensure there is no allowable movement up or down.

3.3 Fresh Frozen Tissue Sectioning

Fresh-frozen tissues for CODEX® analysis should be cut and let adhere directly onto poly-lysine-coated coverslips. Preparation and storage of tissue slices are critical for sample integrity. Given instructions are limited to what is specific to the CODEX® workflow and they are not intended to be a comprehensive guide on how to process and cut tissue sections. Detailed guidance on tissue processing for fresh frozen samples can be found in the sources listed at the end of Appendix B.

Guidelines

Tissues

- Tissues section adhered to Poly-lysine-coated coverslips can be stored at -80°C for up to six months prior to staining.
- It is critical not to exceed a tissue thickness of 10 µm because it can disrupt the autofocusing capabilities of the microscope.
- For best quality CODEX® data, tissue sectioned should be devoid of folds and tears.
- To ensure the integrity of the tissue slices, it is critical that they are not stacked on top of one another after being placed on coverslips.

Pre-Experiment Preparation

Materials NOT Included in Kits:

- Poly-lysine-coated coverslips prepared in section 3.1.
- Cryo/Freezer box with tube inserts prepared in section 3.2.
- Fresh Frozen Tissue
- Aerosol Spray
- Dry Ice
- Polystyrene container for Dry Ice
- Blade for Tissue Sectioning (we recommend 63069-LP Low Profile Microtome Feather® Blade by Electron Microscopy Sciences)

Prepare Cryostat Chamber

Standard cryostats with temperature control are recommended for producing tissue slices. Most tissues will be sliced in temperature ranges from -15°C to -25°C. The exact temperature is unique to each tissue and needs to be selected according to standard slicing procedures.

Fresh Frozen Tissues - Sectioning Instructions

a. Set the cryostats chamber to tissue-specific temperature range.
b. Place prepared tissue slicing storage box in cryostats chamber to equilibrate at the selected cryostat temperature.
c. Once the cryostat reached the selected temperature, transfer the tissue from the -80°C freezer to the cryostat. Use a container filled with dry ice for transporting the tissue block.
d. Use an aerosol spray to clean coverslips from dust and lint prior to use.
e. Place the Poly-lysine-coated coverslips prepared previously in a cryostat chamber to equilibrate for approximately 20-30 seconds.
f. Slice the tissue between 5-10 µm thick.

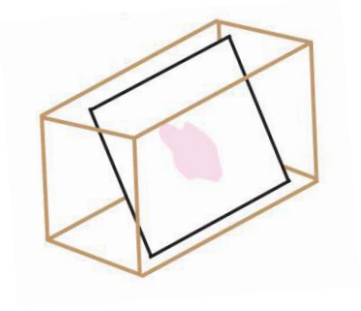
CRITICAL Do not exceed 10 µm because it can disrupt the autofocusing capabilities of the microscope. Avoid folds and tears because they will affect proper data analysis.

g. Gently place the tissue slice in the center of the coverslip as shown in the illustration below.
h. Adhere the sliced tissue to the coverslip by placing a gloved finger on the underside of the coverslip just below the tissue for 1-2 seconds.

CRITICAL Do not keep your finger on the coverslip for more than the minimum time necessary to quickly melt OCT.

Note *This directed heat transfer should effectively melt the OCT and tissue, thereby ensuring adherence. Chemical fixation will take place during the staining protocol.*

i. Place the tissue slice in an individual slot of the prepared tissue slice storage box.



j. Repeat steps d-g for each tissue slice.
k. Once complete, cover the tissue slice storage box with the lid.
l. Place the box of tissue slices on dry ice and transport to a -80°C freezer.

STOPPING POINT

- Samples can be stored at -80°C for up to six months prior to staining with care not to tip container. Make sure the container stays upright.

Note *Tissue processing and sectioning are critical processes and need to be performed by trained users. Resources for best practice procedures and recommendations for avoiding artifacts can be found in Appendix B.*

3.4 FFPE Tissue Sectioning

FFPE tissues for CODEX® analysis must adhere directly onto Poly-lysine-coated coverslips. Preparation and storage of tissue slices are critical for sample integrity. Given instructions are limited to what is specific to the CODEX® workflow and they are not intended to be a comprehensive guide on how to process and cut tissue sections. Detailed guidance on tissue processing for fresh frozen and FFPE samples can be found in the sources listed at the end of Appendix B.

Guidelines

Tissues

- FFPE tissues sectioned onto poly-lysine-coated coverslips can be stored at 4° C for up to six months prior to staining.
- It is critical not to exceed a thickness of 10 µm because it can disrupt the autofocusing capabilities of the microscope.
- For best results, the tissue should be devoid of folds and tears.
- To ensure the integrity of the tissue slices, it is critical that they are not stacked on top of one another after they have been placed on the coverslips.

Pre-Experiment Preparation

Materials NOT Included in Kits

- Poly-lysine-coated coverslips prepared in section 3.1 of the User Manual.
- Cardboard freezer box with tube inserts prepared in section 3.2 of the User Manual.
- FFPE tissue block
- Blade for Tissue Sectioning (we recommend using 63069-LP Low Profile Microtome Feather® Blade by Electron Microscopy Sciences)
- Aluminum Foil
- Aerosol Spray
- 40°C water bath
- Clean Surface

Prepare Microtome

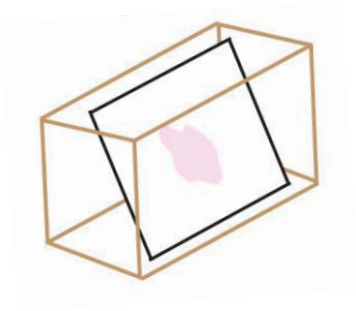
Prepare the Microtome of choice for use at room temperature following the standard procedures of the instrument.

FFPE Tissues - Sectioning Instructions

a. Prepare a water bath at 40°C and place it next to the Microtome.
b. Prepare a clean, dry surface for laying the coverslips next to the Microtome.
c. Use an aerosol spray to clean coverslips from dust and lint prior to use.
d. Place the poly-lysine-coated coverslips next to the Microtome.
e. Insert a new blade for sectioning each block
f. Section the tissue between 5-10 µm thick.

CRITICAL Do not exceed 10 µm because it can disrupt the autofocusing capabilities of the microscope. Avoid folds and tears because they will affect proper data analysis.

g. Place the sectioned tissue in the water bath for a few seconds and observe it expanding.
h. When the observed expansion is enough to remove folds and wrinkles from the tissue, using the forceps, quickly place a Poly-lysine-coated coverslip in the water bath and gently move it upward towards the tissue. Doing so, the tissue will lay on the coverslip as this is moved out from the water bath.
Make sure that the tissue slice is located at the center of coverslip.
i. Put the coverslip on a clean surface with the tissue facing up and let it air-dry overnight.
j. Repeat steps d-g for each slice of tissue.
k. When the tissues are dry, place each tissue coverslip in an individual slot of the storage box and cover the storage box with the lid.
The box of tissue slices can be kept at 4°C for up to 6 months.



STOPPING POINT

- Samples can be stored at 4° C for up to six months prior to staining with care not to tip container. Make sure the container stays upright.

Appendix B. Artifacts of Tissue Processing

General Considerations

Tissue dissection, processing and embedding are very critical for the preservation of tissue morphology and for the quality of the staining. We highly recommend that these procedures are performed only by trained users.

The purpose of this appendix is to illustrate some tips for avoiding common artifacts in fresh-frozen tissue sections and it is not intended as a comprehensive manual for tissue processing and sectioning. An exhaustive technical guide and some useful links on processing fresh frozen [1-3] and FFPE [4,5] sections are indicated at the end of this sections.

General Best Practice Tips for Tissue Sectioning

- Place the tissue block in a container full of dry ice when transferring the tissue block from the -80 C freezer to the cryostat
- Make sure that the cryostat reached the selected temperature before transferring the tissue
- Before starting tissue sectioning, make sure to equilibrate the tissue at the selected cryostat temperature for at least 20 minutes
- We recommended to use 63069-LP Low Profile Microtome Feather® Blade by Electron Microscopy Sciences. Please consider that each sectioning session requires a new blade to avoid tears
- Fatty tissues as lymph-nodes and breast samples require the blade to be wiped with Ethanol to remove residual fat every few sections

A comprehensive report on artifacts of fresh-frozen tissues can be found in [1], where pictures related to freezing artifacts can also be found.

Artifacts, Causes and Tips

1. **Tissue disruption when Sectioning**

Causes: Nicked Blade. Composite samples as fatty tissues (e.g. lymph nodes, breast, skin) might be difficult to cut owing to the presence of fat.

Tips:

- Use a new cutting blade in each sectioning session
- Choose the temperature of the cryostat ideal for the specific tissue of interest
- Wait longer for the tissue to equilibrate at the cryostat temperature if it results too firm for sectioning

2. **Holes**

Causes: Autolyisis*, formation of ice crystals (refer to point 3), over or under-freezing.

**Autolysis is the process of cell self-digestion (and consequent destruction) by the action of its own enzymes. It is caused by the delay between the time when the tissue becomes anoxic and when it is fully frozen.*

Tips:

- Minimize the time between tissue dissection and freezing
- Piling dry ice over the tissue before dissecting it from the animal can also help
- For *over-freezing*: polish the block with a couple extra turns of the blade to create friction and warm up block by pressing on it with your finger (5 - 10 seconds)
- For *under-freezing*: Make sure the cryostat reached the selected temperature before transferring the tissue, in some cases adding a heat sink to block can help

3. Ice crystals and Swiss Cheese Appearance

Causes: The tissue freezing is too slow and gives water the time to crystallizes. Large ice crystals can rupture cell membranes causing the formation of holes.

Tips:

- Freeze tissues faster (3 or 4 seconds to solid) to prevent water for crystallize (it will turn into a vitreous form instead). This might require immersion in -80 C or colder fluid, or complete embedding in powdered CO_2. Cold (-80 C) isopentane is recommended for full tissue immersion
- Do not freeze fat issues surrounding the tissue of interest
- Work with smaller tissues - dimensions should be smaller or equal to 0.5 x 0.5 x 0.3 cm
- Do not use tissues larger than the diameter of the chuck
- Dry the surface of the tissue by pressing with a gauze before making the block
- Once frozen, put a little refrigerated (4 C) OCT on a refrigerated pedestal, then drop the tissue on it and dip the pedestal to quickly freeze the OCT
- After freezing do not let tissue be at -20 C for more than an hour

4. Air Bubbles

Causes: Air bubbles can be trapped under cover slips can cause the tissue to dry out.

Tips:

- Keep the coverslips in the cryostat
- Gently move air bubbles off the slide with finger or tweezers
- Some users find it helpful to place the section directly on the zone of the slide where the finger is placed on the back. This difference in temperature may allow a good spread of the section
- Moving the slide up and down can also help spreading the tissue

Useful Resources

[1] S.R. Peters (ed.), *A Practical Guide to Frozen Section Technique*, DOI 10.1007/978-1-4419-1234-3_2, © Springer Science + Business Media, LLC 2010
[2] https://www.feinberg.northwestern.edu/research/docs/cores/mhpl/tissuefreezing.pdf
[3] http://www.ihcworld.com/_protocols/histology/frozen_section_technique_1.htm
[4] https://www.agilent.com/cs/library/technicaloverviews/public/08002_ihc_staining_methods.pdf
[5] F.L Carson, C.H. Cappellano*, Histotechnology: A Self-Instructional Text*, 4th Edition, ISBN: 9780891896319, ASCP 2015