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Collecting, freezing and shipping frozen post mortem skeletal muscle for single nuclear and spatial RNAseq

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We are still developing and optimizing this protocol

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Abstract

Collecting and freezing skeletal muscle tissue from a clinical site for experimentation at a remote location can be a logistical challenge. This protocol provides the procedures for collecting, snap freezing and shipping frozen skeletal muscle autopsy samples from the morgue to a remote laboratory site for downstream experiments requiring preserved muscle histology and high-quality RNA such as single-nuclear and spatial transcriptomics, and immunofluorescence

Guidelines

Tissue collection for this protocol needs prior approval by the users' Institutional Ethics Board or equivalent ethics committee.



Materials

	A	B	C	D	E
	Organ Transplant Solution	Beltzer UW cold storage solution	Fisher Scientific, cat# NC2122383	Bridge to life	https://bridgetolife.com/belzer-uw-cold-storage-solution/
	RNase Inhibitor	Protector RNase inhibitor	Millipore Sigma # RNAINH-RO	Roche # 3335402001	https://www.sigmaaldrich.com/US/en/product/roche/rnainh-ro?gclid=EAlaQobChMI9e200ZeDgQMVcc7lCh3dKAagEAAYAiAAEgIPpPD_BwE
	Cryotubes	Corning™ Internally Threaded Cryogenic Vials	Fisher Scientific, # 03-374-21	Corning # 430488	https://www.fishersci.com/shop/products/corning-internally-threaded-cryogenic-vials-15/0337421?crossRef=337421#?keyword=337421
	Scale / balance (milligram units)	Laboratory grade balance; OHAUS Pioneer PX Precision Balances	Fisher Scientific, # 01-922-178	OHAUS # 30429848	https://www.fishersci.com/shop/products/pioneer-px-balance-26/01922178?keyword=true
	Small Forceps	Small forceps for handling tissues	Fisher Scientific, # 13-812-211		
	Large Tweezers	Large stainless-steel tweezers,	Cole-Parmer Essentials,		



	A	B	C	D	E
		Straight Narrow Tip, Serrated 305 mm / 12 in	# UX- 07288-14		
	Kimwipes	Small tissues made by Kimberly- Clarke	Fisher Scientific, Catalog # 06-666		
	Freezer packs	Sonoco ThermoSafe PolarPack Standard	Fisher Scientific, # 03-530-110		
	Insulated foam shipping kit	Insulated foam box fitted inside a cardboard shipping box, at least 12 × 12 x 11.5 inches	Uline, # S- 13392		
	Ice Bucket or insulated foam box	Fisherbrand ™ Polyurethan e Ice Buckets	Fisher Scientific, #02-591-45		
	Weigh boats	Small (1.75" x 1.75" x 0.24") and medium weigh boats (3.25" x 3.25"x 1")			
	OCT	Optical cutting temperature (OCT) compound	VWR # 25608-930	Tissue-Tek # 4583	
	Small stainless- steel beaker	250mL metal beaker for submerging isopentane into liquid nitrogen dewar	VWR # 89075-592	Ace Glass # 10300-08	
	Small rope	Braided rope (2-3 mm diameter kevlar or	Find at hardware store		



	A	B	C	D	E
		polyester) rope			
	Liquid nitrogen dewar Flask (1L)	Nalgene Polyethylen e Dewar Flask, Electron Microscopy Sciences	VWR, # 100497-540	Ace Glass # 10300-08	
	Isopentane	AKA 2- methyl butane, use to snap freeze tissue	Sigma Aldrich, # M32631- 500ML		
	Razorblade or surgical knife	Clean and sharp to cut through muscle			
	Wet Ice				
	Dry Ice				
	Aluminum Foil				
	Scissors				
	Gloves				
	Liquid Nitrogen				
	Packaging Tape				
	Shipping label				



Prior to freezing

- 1 Collect the human muscle autopsy samples, keeping them moist in transplant solution or saline, weigh and divide the muscle into 250mg pieces or less beforehand.

Note

- Please refer to the sample collection steps in the "Collection and shipment of live skeletal muscle autopsy for RNA and cell isolation" (see steps 1-8; DOI: [dx.doi.org/10.17504/protocols.io.5qpvo3krzv4o/v1](https://doi.org/10.17504/protocols.io.5qpvo3krzv4o/v1))

Freezing the muscle samples

- 2 Prepare several items and reagents and place on laboratory bench
 - 2.1 Fill a foam box with dry ice. Cover with lid.
 - 2.2 Fill the dewar with liquid nitrogen and cover.
 - 2.3 Cut aluminum foil into 2cm x 3cm rectangular pieces. Loosely fold the foil into One per 250mg muscle.
 - 2.4 Tie the braided rope around the stainless-steel beaker so it can be safely lowered into the liquid nitrogen dewar
 - 2.5 Label the cryotubes with sample ID, current date and other relevant information.
 - 2.6 Cut a small hole in the top of the OCT bottle and place on bench
- 3 Fill 1/3 of the stainless-steel beaker with isopentane and carefully lower into the liquid nitrogen dewar.

Note

The isopentane is ready for snap freezing tissue when it is nearly -160°C or when it starts to form a white frozen layer along the edges of the of the beaker. It should look like a white well of liquid isopentane.

- 4 Add creases to the foil by pre-folding the foil rectangles into thirds, by folding the foil up along the long side using the small forceps.

Note

The foil should be in the shape of a long trough that when loosely wrapped around the muscle like a tube can fit into the mouth of the cryotube.

- 5 Take out one of the cut muscle pieces from the transplant solution and place onto kim wipes.

- 6 Thoroughly dab and dry off the excess liquid from the muscle with additional wipes (approximately as dry as a fresh sample)

Note

If there is excess liquid prior to freezing, the muscle may appear to have swollen fibers, and have abnormally large interstitial spaces when observed under the microscope. They may be prone to freeze artifacts as well.

- 7 Add a tiny dab of OCT into the middle/bottom of the foil trough and place the dried off muscle onto the OCT lengthwise.

Note

Too much OCT will prevent the frozen muscle sample from fitting into the cryotube

- 8 Loosely wrap the sides of the foil along the creases to form a tube around the muscle piece and pinch the foil at one of the long ends. Careful not to crush the muscle.

**Note**

Do not press the foil or wrap too tight. The foil should only be used to hold the muscle and allow it to retain its natural shape while allowing it to fit into a cryotube after frozen.

- 9 Using the long tweezers, carefully grab the foil wrapped muscle sample by the pinched-off end and submerge the sample into the well of liquid isopentane for 20-30 seconds to snap freeze.
- 10 Pull the sample out of the isopentane, shake off the excess, slide into the labeled cryotube and place on dry ice.
- 11 Repeat steps 4-10 until all muscle pieces are frozen and kept on dry ice.
- 12 Pull the beaker out of the liquid nitrogen dewar, allow it to thaw and pour the isopentane back into the original bottle for reuse.
- 13 The snap frozen samples can be stored at -80°C for further use.

Preparing the samples for shipment

- 14 Prepare shipping material by obtaining an insulated foam box fitted inside a cardboard shipping box.
- 15 Fill the foam box with dry ice, place the sample tubes inside, and close the box
- 16 Close the insulated foam box and tape the outer cardboard box layer shut. Label and send it out for overnight shipping.



Expected result

We have tested the RNA quality of human autopsy samples collected at various postmortem intervals (6-72 hours), snap frozen and shipped to a remote laboratory site. The samples were snap frozen in liquid nitrogen cooled isopentane at the sample collection site, sectioned on a cryostat and RNA extracted using a Zymo RNA isolation kit. The whole muscle RNA quality was assessed on an Agilent TapeStation. Samples had RIN numbers ~8. The frozen muscle should be suitable for single-nuclear extraction, histological examination and spatial transcriptomics.

The protocol has been successfully used for mouse skeletal muscle. RNA isolated from mouse muscles processed this way had RIN > 7.5. The snap frozen tissues were used for mouse spatial transcriptomics (Visium) and single-nuclear RNA-seq.