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morphoFISH Perfusion

Forked from a private protocol

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https://dx.doi.org/10.17504/protocols.io.n92ld8d67v5b/v1

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Protocol status: Working
We use this protocol and it's working

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Keywords: mrna transcript, rna, maximal retention of rna, crosslinking rna, tissue clearing, proteins to the scafold, perfusion, transcript, long histological procedures such as tissue clearing, embedding hidrogel, fixation, protein, fixation protocol, tissue, fixative

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Abstract

morphoFISH perfusion is a fixation protocol aimed at maximal retention of RNA in the mby crosslinking RNA and proteins to the scafold formed by fixatives and an embedding hidrogel. As a result of the high retention, mRNA transcripts can be detected after long histological procedures such as tissue clearing.

Materials

MATERIALS

Phosphate Buffered Saline **Thermo Fisher Scientific** **Catalog #**28374

(PBS)

Falcon Tube (50 mL) **Fischer Scientific**

Paraformaldehyde (prilled 95%) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**441244

N,N,N′,N′-Tetramethylethylenediamine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T7024

(TEMED)

4-Hydroxy-TEMPO **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**176141

(4HT)

Nuclease-free water or water filtered using a Milli-Q filtering system **Ambion** **Catalog #**AM9932

MOPS pH 7 **Thermo Fisher Scientific** **Catalog #**AAJ61821AK

N-N-Bis(acryloyl)Cystamine **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A4929-5G

(BAC)

40% Acrylamide Solution **Bio-Rad Laboratories** **Catalog #**1610140

Methacrylic acid N-hydroxysuccinimide ester **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**730300-1G

(MA-NHS)

DMSO, Anhydrous **Thermo Fisher** **Catalog #**D12345

Ammonium Persulfate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**A3678

(APS)

RNase AWAY™ Surface Decontaminant **Carl Roth** **Catalog #**A998.4

Equipment

Standard Pattern Forceps	NAME
Tools	TYPE
Fine Science Tools	BRAND
11001-12	SKU
https://www.finescience.com/en-US/Products/Forceps-Hemostats/Standard-Forceps/Standard-Pattern-Forceps/11001-12	LINK

Isoflurane **Penn Veterinary Supply, Inc. Catalog #**CED1360

Equipment

Surflo winged infusion set 21G 3/4	NAME
Terumo	BRAND
TER 3SV-21BLK	SKU

Equipment

Surgical Scissors	NAME
Fine Science tools	BRAND
14090-09	SKU
https://www.finescience.com/en-US/Search?searchtext=14090-09	LINK

Equipment

Forceps

NAME

Fine Science tools

BRAND

11223-20

SKU

https://www.finescience.com/en-US/Products/Forceps-Hemostats/Dumont-Forceps/Dumont-2-Laminectomy-Forceps/11223-20

LINK

Equipment

Tygon E-1000 Tubing, 1/8" ID x 1/4" OD; 50 Ft

NAME

tubing

TYPE

Tygon

BRAND

EW-50106-11

SKU

https://www.coleparmer.com/i/tygon-e-1000-tubing-1-8-id-x-1-4-od-50-ft/5010611

LINK

Equipment

Watson-Marlow 120S Variable-Speed Single-Channel Pump System	NAME
Peristaltic pump	TYPE
Watson Marlow	BRAND
EW-50130-00	SKU
https://www.coleparmer.com/i/watson-marlow-120s-d1-variable-speed-pump-system-manual-control-1-to-200-rpm-single-channel-4-roller-90-to-264-vac/5013000	LINK

Recipes

	A	B	C	D	E
	4HT	0.5 wt%	50mg	9.95mL water	store -20°C, several months
	TEMED	10 vol%	50uL	450uL water	store single use aliquotes at -20°C, several months
	APS	10 wt%	50mg	450uL water	store single use aliquotes at -20°C, several months

	A	B	C	D	E	F
	1	1M MA-NHS	1M	186.3mg	1mL anhydrous DMSO	Make fresh day of perfusion
	2	BAC	20%	120mg	600ul anhydrous DMSO	Make fresh day of perfusion

	A	B	C	D	E
	Melphalan	2mg/mL	250mg	125mL anhydrous DMSO	store single use aliquots at -20°C

Make solution on a hot plate with a stir bar. Mix for 02:00:00 or until dissolved at 37 °C until completely dissolved. Aliquot (single use aliquots, i.e., 50mL/perfusion) and store at -20 °C for two months. Discard if turns yellow in color.

Note

Melphalan is highly reactive. Variability between lots is expected if e.g., the container overheated or was mishandled during delivery. Being a nitrogen mustard, it can react with itself, water, etc. To minimize such variability, dissolve exclusively in anhydrous DMSO, and monitor color changes in the solution. Open container only once and use only single-use frozen aliquots.

	A	B	C	D	E
	Perfusion fixative				
				tot vol:	50 mL
		Stock	final		volume (mL)
	32% PFA (%)	32	4		6.25
	Nuclease free water				43.75

	A	B	C	D	E
	Protein anchoring solution				
				tot vol:	50
		Stock	Final		Volume
	PFA (%)	32	4		6.25
	Nuclease free water				42.7
	MA-NHS (mM)	1000	20		1

	A	B	C	D	E
				tot vol:	50
	Gel solution				
		stock	final		volume
	AcAm (%)	40	20		25

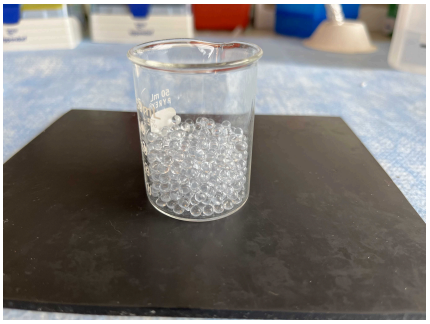
	A	B	C	D	E
	BAC (%, in DMSO)	20	0.1		0.25
	PB pH7.5 (mM)	1000	100		5
	PFA (%)	32	4		6.25
	TEMED pH7.5 (%)	10	.2		1
	4HT (wt%)	.5	0.01		1
	APS (wt%)	10	.2		1
	water				10.5

	A	B	C	D	E
	A	B	C		D
	Post-fixation solution				
				Tot vol:	50
		Stock	Final		Volume (mL)
	PFA (%)	32	4		6.25
	Glutaraldehyde (%)	10	.05		.250
	MOPS pH 7 (mM)	1000	10mM		1
	Melphalan (mg/mL)	2	1		25
	Nuclease free water				17.5

Prepare solutions and set up fume hood for perfusion

1 Thaw aliquoted reagents to room temperature and prepare perfusion buffers: perfusion fixative, protein anchoring solution, gel solution. 1h

2 Prepare 50 mL beaker 1/3 filled with 5 mm glass beads. Rinse both with RNase AWAY™ prior to use.



3 Set up fume hood with dissection tray, perfusion pump, 4 50 mL conical tubes containing the perfusion solutions, forceps, scissors, and biohazard bag. Clean all tools with RNase AWAY™ prior to use. 15m

Sequential Perfusion

1d 17h 30m

4 Prepare perfusion pump by attaching butterfly needle to the tubing. Start the pump and flush aprox. 20 mL of 1x RNAse-free PBS to wash tubing. Stop the pump. Make sure no bubbles are in the line.

5 Retrieve animal per vivarium protocol.

6 Prepare anesthesia chamber by pouring aprox. 5 mL isoflurane into the bottom of desiccation jar and placing a paper towel on the porcelain insert. 1m

6.1 Place the animal inside the container on the insert and cover. 3m

Note

Do not allow liquid isoflurane to come into contact with the animals' skin as this can cause irritation.

6.2 Monitor breathing. Once the mouse respiration reduces to 2 breaths per second, check for plantar reflex by gently pinching the foot with forceps. If no reaction, remove the mouse from the jar and place on dissection tray. If the mouse reacts close the lid and wait ~30seconds. Check reflex again until no reflex observed.

Note

Timing is extremely important. Over-exposure to isoflurane can lead to cardiac arrest. Premature cell death and blood clots will impact the quality of the perfusion

7 Begin perfusion.

Note

When transitioning between perfusion solutions make sure to stop the pump prior to moving intake tubing to prevent bubbles from forming. Perfusing air bubbles can cause major deformations, including in brain ventricles.

7.1 Lay mouse on its back and place a pin in each front paw, others as needed.

7.2 Carefully use scissors to open the chest cavity by lifting the base of the sternum and cutting parallel to either side of the sternum to the neck, then cut the diaphragm without puncturing the heart or lungs. Expose the heart.

7.3 Use forceps to gently hold the heart by the right ventricle. Insert the tip of the butterfly needle into the bottom apex of the left ventricle. Cut the right atrium to allow for drainage

NB:
- (Optional) Use a 18G needle to secure the butterfly needle in place or a hemostat clamp.
- Correct placement of the needle is important. Do not push the needle too deep. Only insert the tip of the needle just past the beveled angle of the tip.

7.4 Start perfusion pump and flush 10 mL 1xPBS at a rate of 7.5mL/min.

> Note
>
> Liver should turn pale tan color during. If the liver does not become lighter, check needle position to ensure it did not puncture through the ventricle wall.

7.5 Stop perfusion pump. Transfer intake tubing to the conical tube with the first perfusion solution, i.e., [M] 4 % (v/v) PFA. Start the pump and flush 50 mL

10m

NB:
- Muscle contractions are poised to occur. If not, check placement of the needle and reposition as needed
- Intestines and liver may extend outside of the abdominal cavity and displace needle

7.6 When only 10 mL of fixative remains, add 1 mL 1M MA-NHS to anchoring solution. Mix well by inverting 5x. Stop perfusion pump and transfer intake tubing to anchoring solution freshly supplemented with MA-NHS. Start the pump and flush 50 mL of protein anchoring solution

10m

7.7 When only 10 mL of protein anchoring solution remains, add 1 mL 10% TEMED, 1 mL 0.5% 4HT, and 1 mL 10% APS to *gel solution*. Mix well by inverting x5. Stop perfusion pump and transfer tubing to gel solution. Start the pump and flush 50 mL gel solution

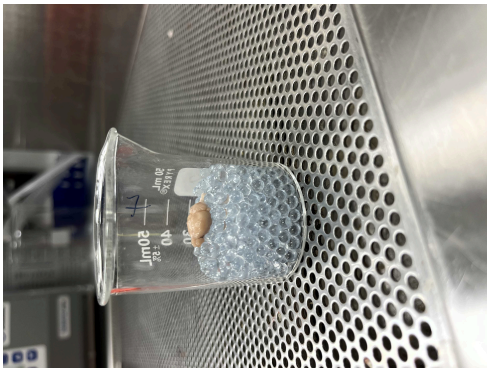
10m

7.8 Stop the pump and remove the needle from the ventricle. Flush the tubing with 20 mL PBS to purge gel solution.

8 Remove animal from dissection tray.

8.1 Dissect out the brain.

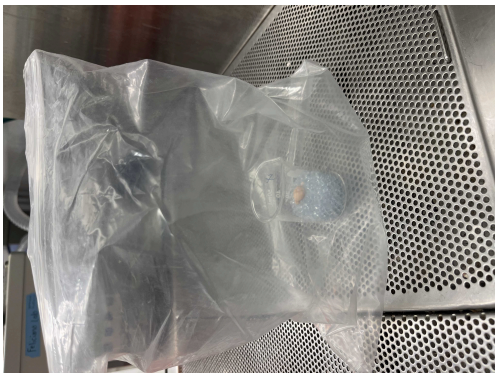
9 Polymerize perfused gel by placing the brain in the beaker with glass beads (ventral side down). Place beaker in sealable plastic bag.



9.1 Remove oxygen from the bag by filling the bag with Nitrogen gas from a compressed tank. Replace bag's atmosphere a second time by compressing bag and refilling with Nitrogen gas.

NB: The Nitrogen source should be near the dissection table so that the gel can polymerize as quickly as possible.

9.2 Seal bag and gel for 01:00:00 at Room temperature 1h



10 Prepare 50 mL of *post-fixation* solution. Transfer brain to post-fixation solution for 40:00:00 at 40 rpm, 4°C

1d 16h

10.1 Rinse 3x with RNAse free PBS for 00:30:00 , at 40 rpm, Room temperature

30m

Brain can be stored in 1x PBS @ 4C for further processing. For optimal detection of RNA it is recommended to avoid delays to prevent RNA degradation.