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iDISCO Clearing and Staining of Pancreas

Forked from iDISCO Clearing and CTb staining of DRGs, Coealic and Nodose

Maria Jimenez Gonzalez[1], Sarah Stanley[1], Rosemary Li[1]

[1]Icahn School of Medicine at Mount Sinai

1 Works for me dx.doi.org/10.17504/protocols.io.baxbifin

Rosemary Li
Icahn School of Medicine at Mount Sinai

ABSTRACT

This is a protocol for iDISCO Clearing and immunostaining of pancreata/intrapancreatic ganglia in mice.

GUIDELINES

This study was approved by the Institutional Animal Care and Use Committee of Icahn School of Medicine at Mount Sinai

MATERIALS

NAME	CATALOG #	VENDOR
Normal Donkey Serum	017-000-121	
Methanol	M3641	Sigma Aldrich
Paraformaldehyde, 16% (wt/vol)	15710	Electron Microscopy Sciences
Triton X-100	T8787	Sigma Aldrich
Hydrogen Peroxide, 30%	H325-500	Fisher Scientific
Heparin	H3393	Sigma Aldrich
Dimethyl Sulfoxide (Certified ACS) Fisher Chemical	D128-500	Fisher Scientific
Dichloromethane	270997	Sigma Aldrich
Benzyl ether	108014	Sigma Aldrich
Glycine	410225	Sigma Aldrich
Tween-20	P9416	Sigma Aldrich
Sodium azide	S2002	Sigma Aldrich
Isoflurane (Forane)	1001936040	Baxter
Agarose	A7705	Alkali Scientific

MATERIALS TEXT

Rabbit anti-HA-tag mAb, Cell Signaling Technologies, Cat# 3724, RRID:AB_1549585
Rat anti-Insulin mAb, R&D Systems, Cat#MAB1417, RRID:AB_2126533
Guinea Pig anti-Insulin pAb, Dako/Agilent, Cat# 0564, RRID:AB_10013624

Alexa Fluor 647-AffiniPure Donkey Anti-Rabbit IgG (H+L), Jackson ImmunoResearch, Cat# 711-605-152, RRID:AB_2492288
DyLight 550 Donkey anti-Rat IgG (H+L), Thermo Fisher Scientific Cat# SA5-10027, RRID:AB_2556607
Alexa Fluor 488 Goat anti-Guinea Pig IgG (H+L), Thermo Fisher Scientific Cat# A-11073, RRID:AB_2534117

SAFETY WARNINGS

Some chemicals, as DBE and DCM are toxic and corrosive. Always use double gloves and perform the steps that involve these reagents under the fume hood.
Sdium Azide is highly toxic. Wear standard Protective Personal Equipment (Mask, gloves,labcoat) when handling sodium azide powder or solution. It is recommended to double glove or wear thicker nitrile rubber gloves. Remove gloves immediately if contaminated, and wash hands with soap and water.

BEFORE STARTING
Perfusions with PFA should be done under the fume hood

Perfusion

1 Preparation of Buffers
- Heparinized PBS: 10 mg/100 ml PBS
- Prepare a 4% PFA solution in PBS (Dilution of 1:4 of 16%PFA)

2 Place the solutions (Heparanized PBS and 4% PFA) in a box with ice and prime the tubes of the perfusion pump with HeparInized PBS. Anesthetize the mouse by inducing anesthesia with Isoflurane at 3%. One deeply anesthetized, place the mouse in supine position and keep anesthesia at 1-1.5% using an orofacial mask. Open the abdominal cavity, open the diaphragm and place the needle (22-25G) attached to the perfusion pump tube into the left ventricle of the heart. Subsequently, pierce the right atrium with a needle. Start the perfusion at a rate of 5 ml/min. Check that the blood is coming out and that the liver is getting pale. After infusing **20 ml** aproximately, change to the infusion of 4% PFA. After 1-2 min, there must be a 'shrinking' of the body.After infusing **20 ml** of PFA, the perfusion is finished, proceed to harvest the tissues of interests in the microtubes.

Tissue harvest

3 With the use of a dissection microscope, remove the pancreas from the abdomen. Remove fat, lymph nodes, and hair as needed (easily done immersed in PBS, using dissection microscope). Keep in 4% PFA Solution at **4 °C** overnight. Next day, wash 3 times with PBS before starting tissue clearing, sectioning or any other protocol. If tissue clearing, cut pancreas into approximately 8 equal pieces (to facilitate antibody permeabilization).

iDISCO - Tissue clearing

4 Preparation of buffers:

Ptx2 1L:
- Triton X-100 2 ml
- Sodium Azide 0.01%
- PBS 100 mM, pH 7.4

PtwH 1L:
- Tween-20 2 ml
- Heparin 10 mg
- Sodium Azide 0.01%
- PBS 100 mM, pH 7.4

Permeabilization Buffer 0.5L:
- Triton X-100 0.8 ml
- Glycine 11.5 g
- DMSO 100 ml

5 **Dehydrate in an increasing gradient of Methanol/H2O, samples must be at room temperature and shaking at 20-30rpm in a wave-motion shaker:**

20% MetOH for 1h
40%MetOH for 1h
60%MetOH for 1h
80%MetOH for 1h
100% MetOH for 1h
100% MetOH for 1h or overnight (**4 °C**)
66/33% Dichloromethane (DCM)/Methanol for 3h, at room temperature, protecting samples from light
100% MetOH for 1h or overnight (**4 °C**)

6 **Bleaching protocol**

Wash twice in 100% Methanol at room temperature
Chill samples at **4 °C** during **00:15:00**
Bleach samples in freshly prepared 5/95% H2O2/MetOH, overnight at **4 °C**

7 **Rehydrate/Permeabilization in an increasing gradient of Methanol/H2O, samples must be at room temperature and shaking at 20-30rpm in a wave-motion shaker:**
80% MetOH for 1h
60%MetOH for 1h
40%MetOH for 1h
20%MetOH for 1h
The following are permeabilization steps:
PBS for 30 min
Ptx2 for 1h
Change the Ptx2 for 1h
Finally place samples in Permeabilization Buffer for 1 day at **37 °C** and shaking

8 **Blocking Step:**
Place samples in a blocking buffer consisting of: 6%Donkey Serum , 10%Dexamethanose (DMSO) in Ptx2 for 48h at **37 °C** and shaking

9 **Primary Antibody Incubation:**
Primary antibody is diluted in 3%Donkey Serum, 10%DMSO prepared in PtwH. Samples are incubated for 96h at **37 °C** and shaking. Dilution of Rabbit anti-HA is 1:500. Dilution of GP and rat anti-insulins are 1:1000.

10 Wash in PtwH 4-5 times for 1 hour minimun per wash, shaking the samples at room temperature. Leave them in the last wash overnight.

11 **Secondary Antibody Incubation:**
Secondary antibody is diluted in 3% Donkey Serum in PtwH. Samples are incubated for 96h at **37 °C** and shaking. Dilution for all secondaries is 1:500.

12 Wash in PtwH 4-5 times for 1 hour minimun per wash, shaking the samples at room temperature. Leave them in the last wash overnight.

13 **Dehydrate in an increasing gradient of Methanol/H2O, samples must be at room temperature and shaking at 20-30rpm in a wave-motion shaker:**
20% MetOH for 1h
40%MetOH for 1h
60%MetOH for 1h
80%MetOH for 1h
100% MetOH for 1h
100% MetOH for 1h or overnight

14 **Clearing step:**
Place samples in 66/33% DCM/Methanol, 3h incubation at room temparature with shaking. Protect samples from light. Change to 100% DCM for 15 min at room temperature with shaking. Replace the 100% DCM for another 15 min incubation. Place in DBE,and shake samples at room temperature, protecting from light. Samples should look 'transparent' after 30 min - 1 hour. *Note: clean DBE spills with MetOH.*
Samples can be kept in DBE for months. Better store them at **4 °C**

Visualization-Microscopy

15 For visualization of these samples, confocal microscopy is sufficient, we use Zeiss LSM780 microscopes:
- Upright microscope: Place the samples in 3D printed VisiJet samples (https://idiscodotinfo.files.wordpress.com/2015/04/whole-mount-staining-bench-protocol-methanol-dec-2016.pdf)
- Inverted microscope: Place the samples in glass bottom Slides (e.g. ibidi, cat#80827)

Analysis and Quantification

16 Acquired images (e.g. in czi format) are converted and visualized in IMARIS software. Using the 3D Spot object quantification, we quantified the positive stained neurons and total neurons.