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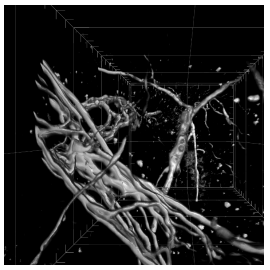
Human Pancreas PACT Optical Clearing and High Resolution 3D Microscopy



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We use this protocol and it's working

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Disclaimer

Abstract

Using traditional histological methods, researchers are hampered in their ability to image whole tissues or organs in large-scale 3D. Histological sections are generally limited to <20 µm as formalin fixed paraffin section on glass slides or <500 µm for free-floating fixed sections. Therefore, extensive efforts are required for serial sectioning and large-scale image reconstruction methods to recreate 3D for samples >500 µm using traditional methods. In addition, light scatters from macromolecules within tissues, particularly lipids, prevents imaging to a depth >150 µm with most confocal microscopes. To reduce light scatter and to allow for deep tissue imaging using simple confocal microscopy, various optical clearing methods have been developed that are relevant for rodent and human tissue samples fixed by immersion. Several methods are related and use protein crosslinking with acrylamide and tissue clearing with sodium dodecyl sulfate (SDS). Other optical clearing techniques used various solvents though each modification had various advantages and disadvantages. Here, an optimized passive optical clearing method is described for studies of the human pancreas innervation and specifically for interrogation of the innervation of human islets.

Attachments

PDF

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786KB

Materials

	Name of Material/Equipment	Company	Catalog Number	Comments		
	10x phosphate buffered saline (PBS)	Fisher	BP399-1	Buffers		
	Sodium phosphate dibasic anhydrous	Fisher	S375-500	PB buffer (RIMS)		
	Sodium phosphate monobasic monohydrate	Sigma	71507-250	PB buffer (RIMS)		
	16% paraformaldehyde (PFA)	Electron Microscopy Sciences	15714-5	Immersion fixation, hydrogel, storage solution		
	40% acrylamide	Bio-Rad	161-0140	Hydrogel		
	2% bis-acrylamide	Bio-Rad	161-0142	Hydrogel		
	VA-044 initiator	Wako Pure Chemical Industries, Ltd.	VA044	Hydrogel		
	Sodium dodecyl sulfate (SDS)	Fisher	BP166-5	Clearing buffer		
	Sodium azide	Sigma	S8032	Sample storage buffer		
	18 gauge needles	Fisher	14-840-91	Degassing hydrogel solution		
	N2 tank	AirGas	various	Degassing hydrogel solution		
	Triton X-100	Sigma-Aldrich	100 ml	Buffers		
	Goat, normal serum	Vector	S-1000	Use as 2% in blocking buffer		
	Histodenz	Sigma	D2158-100G	RIMS		
	8-well chamber slides	Ibidi	80827	Imaging		

	Laser scanning confocal microscope	Zeiss	710	Imaging		
	LightSheet microscope	Zeiss	Z1	Imaging		
	Primary Antibody	Host	Vendor	Cat. #	Dilution	Comments
	CD45	Rabbit	Bioss	bs-4820R-A488	1:100	Did not work
	CD45	Mouse	DAKO	M0754	1:200	Did not work
	GFAP	Rabbit	DAKO	Z0334	1:50	Worked
	Glucagon	Mouse	BD Biosciences	565891	1:50	Worked
	Glucagon	Rabbit	Cell Signaling	2760S	1:200	Did not work
	Glucagon	Mouse	Abcam	ab10988	1:200	Worked
	Insulin	Guinea Pig	DAKO	A0564	1:200	Worked
	NCAM (CD56)	Mouse	DAKO	M730429-2	1:50	Did not work
	NCAM (CD56)-FITC conjugate	Mouse	DAKO	M730429-2	1:50	Did not work
	Peripherin	Rabbit	EnCor	RPCA-Peri	1:100	Worked
	PGP9.5	Rabbit	DAKO	Z5116	1:50	Did not work
	Secretogranin 3	Rabbit	Sigma	HPA006880	1:200	Worked
	Smooth muscle actin	Mouse	Sigma	A5228; C6198 (Cy5)	1:200; 1:200	Worked; Conjugated worked better than unconjugated
	Substance P	Rat	BioRad	8450-0505	1:200	Worked

	Tyrosine Hydroxylase	Rabbit	Millipore	AB152	1:200	Worked
	Tyrosine Hydroxylase	Chicken	Abcam	Ab76442	1:100	Worked, but weak staining
	Vasoactive Intestinal Peptide (VIP)	Rabbit	Immunostar	20077	1:100	Worked
	Vesicular Acetylcholine Transporter (VAChT)	Rabbit	Synaptic Systems	139103	1:50	Worked
	Secondary Antibody					
	Guinea pig IgG	Goat	Thermo Fisher Scientific	Various	1:200	AlexaFluor conjugates
	Mouse IgG	Goat	Thermo Fisher Scientific	Various	1:200	AlexaFluor conjugates
	Rabbit IgG	Goat	Thermo Fisher Scientific	Various	1:200	AlexaFluor conjugates
	Rat IgG	Goat, Donkey	Thermo Fisher Scientific	Various	1:200	AlexaFluor conjugates
	Chicken IgG	Goat	Thermo Fisher Scientific	Various	1:200	AlexaFluor conjugates

Troubleshooting

Safety warnings

Paraformaldehyde, xylene, and acrylamide are toxic irritants. Handle reagents in a fume hood with appropriate personal protective equipment (lab coat, gloves, eye protection). Follow EHS guidelines for all chemical handling and disposal requirements.

Deparaffinization of Formaldehyde-fixed Paraffin Embedded Tissues (If Working with Fresh Tissues, Skip to Step 10)

1 Use a new razor blade or scalpel to cut through the paraffin perpendicular to the surface of the tissue. Cut the paraffin at the edge of the tissue to finish loosening it. Use forceps or a spatula to gently loosen and remove the tissue to be optically cleared. Gently scrape excess paraffin from the tissue using a spatula (Figure 1A).

2 Fill a glass container with xylene (about 30 mL for a 3 × 3 × 3 mm section of tissue) and incubate the tissue in this solution for 24 h at room temperature (RT).

3 Fill another glass container with fresh xylene with the same volume as 1.2.1. Transfer and incubate the tissue in this solution for 24 h at RT.

4 Place 100% ethanol into a conical tube with the same volume as 1.2.1. Transfer and incubate the tissue in this solution for 24 h at RT.

5 Place 95% ethanol into a conical tube with the same volume as 1.2.1. Transfer and incubate the tissue in this solution for 24 h at RT.

6 Place 70% ethanol into a conical tube with the same volume as 1.2.1. Transfer and incubate the tissue in this solution for 24 h at RT.

7 Rinse the tissue in 0.01 M phosphate buffered saline (PBS) and place in 0.01 M PBS in a conical tube to equilibrate for 24 h at RT. Ensure that the tissue is free of paraffin (Figure 1B, right panel).

Prepare 4% Paraformaldehyde (PFA) Fixative

8 Pipette 10 mL 16% PFA into a 50-mL conical tube, add 4 mL 0.1 M PBS, and add 26 mL distilled deionized water (ddH2O). Close the cap and mix briefly.

9 Larger volumes of 4% PFA can be made ahead and frozen in aliquots. Aliquots are good for 1 day at room temperature (RT), one week at 4 °C, and 1 month at -20 °C

Pancreas Fixation

10 Fix the pancreas sample (≤ 1 × 1 × 2 cm) in freshly prepared 4% PFA at 4 °C for 48 h. If the sample is larger than 1 × 1 × 2 cm, use a scalpel or razor blade to dissect into smaller pieces no more than 1 cm thick. Wash tissue sample in three changes of 0.01 M PBS for 2d

at least 15 min each wash and store in 15 mL centrifuge tube in 0.01 % PFA/0.01 M PBS or 0.5 % sodium azide/0.01 M PBS until use.

11 After fixation, section the tissue into 1 - 2 mm thick sections for further processing. Use a vibratome to assist in even sectioning. NOTE: The final number of sections will depend on the size of the starting sample.

Embed Tissue in Hydrogel

12 Prepare 200 mL of the 4 % acrylamide/1 % paraformaldehyde (A4P1) hydrogel monomer solution as follows:

12.1 Place a flask on the ice in a bucket on the top of a magnetic stir plate. Make sure the flask is sitting flat and add a magnetic stir bar.

12.2 Add the following in order: 147.8 mL cold (4 - 8 °C) ddH2O, 20 mL 0.1 M PBS, 20 mL cold (4 - 8 °C) 40 % acrylamide solution, 12.2 mL 16 % PFA solution, and 250 mg VA-044 initiator. Mix the entire hydrogel solution with a magnetic stir bar for at least 10 min and leave the solution on ice for the next step.

13 Place a 15-mL conical tube in the ice next to the flask containing the hydrogel solution. Pipette 14 mL of monomer solution into the tube and add one piece of the 1 - 2 mm thick fixed tissue sample. Then cap the tube.

14 Incubate the sample in monomer solution for 3 days at 4 °C and protect from light. Aliquot any remaining monomer solution and store at -20°C for future use.

Degas the Monomer Solution and Polymerize the Hydrogel

15 Remove oxygen from the hydrogel monomer solution using gaseous N_2[8].

15.1 Prepare a bucket of ice and place the sample in the hydrogel monomer solution on ice.

15.2 Gather 2 - 3, 18-gauge hypodermic needles per sample, paraffin film, and a timer. Connect the tubing to the nitrogen tank so that the nitrogen can flow. While keeping the sample on ice, carefully pierce the cap of a conical tube containing the sample on one side and press one hypodermic needle through until it is under the surface of the liquid monomer solution.

15.3 Use another hypodermic needle to puncture the opposite side of the cap, but do not allow it to become submerged. NOTE: The second needle will vent the tube.

15.4 Connect the tubing from the nitrogen tank to the hypodermic needle submerged beneath the hydrogel and slowly turn on the nitrogen until it is bubbling steadily through the liquid.

15.5 Allow the nitrogen to bubble through the liquid for 10 min.

16 Once the oxygen is removed, quickly remove both needles and cover the cap with a paraffin film to prevent any further exchange of gasses between the tube and the environment. Place the degassed sample in an incubator at 37 °C for 3 h to polymerize the hydrogel.

Tissue Clearing

17 Prepare 500 mL clearing solution (4 % SDS at pH 8.5). To ~300 mL of ddH2O, add 50 mL 0.1 M PBS and 20 g SDS powder while stirring with a magnetic stir bar. Adjust the solution using sodium hydroxide and hydrochloric acid to pH 8.5. Add ddH_2O until the final volume is 500 mL.

18 After polymerization, pour away excess hydrogel and discard it into a chemical waste container. Use a paper towel to gently wipe away hydrogel from the sample and discard into a chemical waste container.

19 Wash the sample in 3 - 5 exchanges of 0.01 M PBS (discard wash fluid into the chemical waste) for 15 min each wash step. Transfer the sample into a 50-mL conical tube with 40 mL of clearing buffer.

20 Incubate the sample in the clearing buffer at 37 °C and change sample to fresh clearing buffer every other day.

20.1 Leave the sample in the clearing buffer for 2 - 8 weeks depending on the sample size (~8 weeks for a 3 mm x 3 mm x 3 mm sample) to ensure proper clearing.

20.2 Monitor tissue clearing and stop when complete. Ensure that the sample is adequately transparent by holding it up to the light to check for proper clearing (usually some tan coloring will remain in the exocrine regions).

NOTE: An over-cleared sample will appear frayed at the edges and the texture will be very soft when picked up with forceps. It is common for the sample to clear unevenly. Also, the tissue will not be fully transparent until placed in mounting media (Insert, Figure 1C).

Multiple Immunofluorescence

21 Wash the samples on a shaker at 60 rpm at RT for one day with 40 mL 0.01 M PBS changing to fresh buffer often (4 - 5 buffer changes in total, 40 mL each wash, changing every h until the final wash). Let the final wash continue overnight at RT.

22 Prepare PACT staining buffer. To 500 mL 0.01 M PBS, add 50 mg sodium azide and 0.5 mL TritonX-100. Mix well.

23 Incubate the sample with primary antibodies.

23.1 Add 2% normal serum (same species as the secondary antibody) to the base PACT staining buffer in a 2-mL flat bottom tube (at least 1mL total volume is recommended per sample/tube).

23.2 Add primary antibody to the 2 % serum/PACT staining buffer. Use approximately 5x the amount of primary antibody for PACT staining as would be used for standard immunohistochemistry (i.e. if an antibody is diluted 1:500 for standard immunohistochemistry, use 1:100 for PACT staining).

23.3 Use a spatula to remove the sample from the wash buffer and dab the excess buffer off onto a paper towel, then place in the tube with a primary antibody solution.

24 Incubate 2-4 days at RT on a shaker at 60 rpm. Wash samples thoroughly at RT on a shaker at 60 rpm in 0.01 M PBS changing to fresh buffer 4-5 times and leaving the final wash on overnight as in step 7.1.

25 Incubate samples with secondary antibodies.

25.1 Add 2% normal serum (same species as the secondary antibody) to the base PACT staining buffer in a 2-mL flat bottom tube (1 mL total volume is recommended per sample/tube).

25.2 Add secondary antibodies at a concentration of 1:200 (5 µl in 1 mL buffer).

NOTE: Small format antibodies are preferred, as well as highly cross-adsorbed antibodies if using more than one primary antibody.

25.3 Use a spatula to remove the sample from wash buffer and dab the excess buffer off onto a paper towel, then place in the tube with a secondary antibody solution.

26 Incubate at RT on a shaker at 60 rpm for 2 days and protect the sample from light.

27 Wash the samples thoroughly, as in step 7.1, at RT on a shaker at 60 rpm in 0.01 M PBS changing to the fresh buffer 4 - 5 times and leaving the final wash on overnight, protect from light during washes.

Mounting Samples for Imaging

28 Prepare the refractive index matched solution (RIMS) buffer.

28.1 Weigh out 11 g of non-ionic density gradient medium (e.g., Histodenz) and carefully transfer to a 50-mL conical tube.

28.2 Add ~5 mL 0.02 M phosphate buffer (PB)8 using a spatula to release air from the powder non-ionic density gradient medium.

NOTE: The solution will be very viscous, mix well.

28.3 Bring the volume to 10 mL using more PB, mix with a spatula and scrape the excess off the spatula into the tube.

28.4 Incubate RIMS at 37 °C until dissolved, invert and gently mix as needed

29 Transfer samples into RIMS. To do so, pipette 1 mL RIMs into a 2-mL flat-bottom tube. Use a spatula to remove the sample from wash buffer and dab the excess buffer off onto a paper towel, then place in the tube with RIMS solution.

29.1 Gently tap the tube to submerge the sample in RIMS. Place samples in RIMS on the bench protected from light at RT for 2 - 4 days before imaging.

30 To image, place a small amount of RIMs into an 8-well coverslip bottom chamber slide. Only add just enough to coat the bottom, more will cause the sample to float making it more difficult to image on an inverted scope. Add the sample to the well and cap the slide for imaging.

Confocal Imaging

31 Select appropriate lasers for the excitation and emission spectra of the fluorophores used to stain the PACT samples. Adjust the settings of the acquisition software so that any overlap between channels is eliminated. Use separate tracks if necessary (when twofluorophores have similar excitation spectra).

32 Set up the image acquisition

32.1 Choose maximum acquisition speed, 16 bit, 4 or more averages, and 1024 × 1024 resolution or better.

32.2 Zoom into the object to be imaged.

NOTE: This will decrease acquisition time to reduce photo-bleaching and also decrease file size and downstream editing.

33 Setup the z-stack

33.1 Select the optimal sectioning for the objective being used. If deconvolution is desired later, use a z-step smaller than optimal such as 1 µm.

33.2 Use the z-stack correction.

33.3 Begin nearest the surface of the tissue and increase the gain as the objective focuses through the z-plane and add
corrections. Do not change the laser settings for the correction!

33.4 Acquire a test stack with one average, 512 × 512 resolution, and maximum acquisition speed. Check the image in 3D to make sure that there is equal brightness throughout the stack for each color before acquiring the final high-resolution zstack.

Lightsheet Imaging

34 Ensure that the samples have been equilibrated in RIMS for imaging for at least 24 h. Ideally, transfer the samples to fresh RIMS in the imaging chamber and allow to equilibrate in the chamber for at least a day.

Equipment

Lightsheet	NAME
Light Sheet Microscopy	TYPE
Zeiss	BRAND
2583000198	SKU
https://www.zeiss.com/microscopy/us/products/imaging-systems/lightsheet-z-1.html	LINK

35 Mount the sample for imaging

35.1 Select the smallest (black) capillary to mount the sample

35.2 Use putty or a dish to hold the sample while applying super glue to the end of the capillary. Glue the tissue to the capillary touching as little a surface of the tissue as possible.

35.3 Insert the sample for imaging.

36 Image using 5X or 25X objectives suitable for optically cleared samples using the pivot scan option. If shadowing or blurring occurs, rotate the sample and try again, or let the sample continue to equilibrate in RIMS.

NOTE: A 1 mm stack can generally be acquired in less than five minutes, depending on the settings.

37 View and edit the image stacks using the image analysis software.