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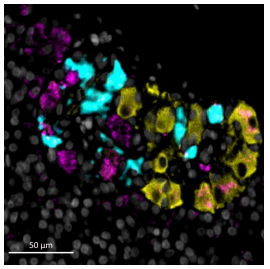
Multiplex Immunofluorescence for Human Pancreatic Ganglia



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We use this protocol and it's working

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Abstract

Purpose:
This protocol is a multiplex immunofluorescence (MxIF) protocol adapted from t-CyCIF (dx.doi.org/10.17504/protocols.io.bjiukkew) and bleach & stain (Bady et al. 2023) protocols. Our protocol involves sequential incubations of primary antibodies using combinations of Opal reagents and fluorescence conjugated secondary antibodies for tyramine signal amplification (TSA) visualization following by standard immunofluorescence for primary antibodies not requiring TSA. Each cycle can consist of 4 or more primary antibodies depending on imaging equipment. Within and between staining cycles, antigen retrieval is performed to strip primary and secondary antibodies. Between staining cycles, a bleaching procedure is included to quench autofluorescence and deposited Opal TSA fluorescence. This protocol can be adapted to include a wide variety of primary antibodies with different combinations of Opal reagents and fluorescence conjugated secondary antibodies.
Expected Outcome:
By carefully selecting combinations of primary antibodies, secondary antibodies, and Opal reagents, one can visualize numerous markers in a single tissue section while avoiding cross-reactivity. As described by Bady et al., this allows for up to 15-20 antibodies to be applied to a single tissue section rather than the typical 4-7 antibodies that can be used in a single run.
Scope:
This protocol can serve as reference source for immunostaining human pancreas endocrine cells and intrinsic autonomic ganglia and is expected to also work well for formalin-fixed paraffin-embedded samples.

Image Attribution

Human pancreatic ganglion stained for ChAT+ neurons (magenta), nNOS+ neurons (yellow), SNAP25+ nerve fibers (teal), and nuclear DNA (DAPI, white).

Materials

Primary and Secondary Antibodies:

MxIF-Antibodies.xlsx 11.8KB

Reagents:

- Histo-Clear II, **Electron Microscopy Sciences Catalog #** 64111-04
- 100% ethanol, **Fisher Scientific Catalog #** 04355223
- 95% ethanol, **Fisher Scientific Catalog #** 04355226
- 70% ethanol, dilute from 100% ethanol, **Fisher Scientific Catalog #** 04355223
- TBST, Tris Buffered Saline with Tween 20, 20X, **Fisher Scientific Catalog #** TA125TT
- PBS, Phosphate Buffered Saline, 10X, **Fisher Stores Catalog #** BP3991
- Antigen Retrieval Buffer (AR9) for Opal TSA, 10x, **Akoya Biosciences Catalog #** AR900250ML (store at 4 °C)
- ImmEdge Barrier PEN (PAP pen), **Vector/Absolute Biotech Catalog #** H4000
- ImmPRESS – HRP Horse Anti-Goat IgG Polymer Reagent, **Vector Laboratories Catalog #** 30036 (store at 4 °C)
- ImmPRESS – HRP Goat Anti-Rat IgG Polymer Reagent, **Vector Laboratories Catalog #** 30032 (store at 4 °C)
- Opal 3-PLEX Manual TSA Kit (520/570/690), **Akoya Biosciences Catalog #** NEL810001KT (store at 4 °C)
- Opal Antibody Diluent/Block, 1X, **Akoya Biosciences Catalog #** ARD1001EA (store at 4 °C)
- Opal Anti-Ms + Rb HRP, 1X, **Akoya Biosciences Catalog #** ARH1001EA (store at 4 °C)
- Opal 1X Plus Manual Amplification Diluent, 50 mL, **Akoya Biosciences Catalog #** FP1498 (store at 4 °C)
- Opal 480-tyramine TSA reagent (AF405), **Akoya Biosciences Catalog #** FP1500001KT (store at 4 °C)
- Opal 520-tyramine TSA reagent (AF488), **Akoya Biosciences Catalog #** OP-001001 (store at 4 °C)
- Opal 570-tyramine TSA reagent (AF555), **Akoya Biosciences Catalog #** OP-001003 (store at 4 °C)
- Opal 650-tyramine TSA reagent (AF647), **Akoya Biosciences Catalog #** FP1496001KT (store at 4 °C)
- Opal 780-tyramine TSA reagent (AF705), **Akoya Biosciences Catalog #** FP1501001KT (store at 4 °C)
- Hoechst S769121 (Nuclear Yellow), dissolve in water to 1:500 and store aliquoted at -20 °C **Abcam Catalog #** ab138903
- Prolong Gold Mounting Media with DAPI, **Thermo Fisher Catalog #** P36931
- Fluoromount-G anti-fade, Aqueous Mounting Media, **Fisher Scientific Catalog #** OB100-01
- 5M NaOH, Sodium Hydroxide, **Fisher Scientific Catalog #** S93369
- Hydrogen Peroxide, 30% (store at 4 °C), **Fisher Stores Catalog #** H325-500 (store at 4 °C)

Supplies and equipment:

- Superfrost Plus Microscope Slides, **Fisher Scientific Catalog #** 1255015

- Coverslips, 22×50mm, No. 1.5, **VWR Catalog #** 48393-195
- Slide Warmer, **Fisher Scientific Catalog #** 12-594
- Food steamer, **Bella Catalog #** XJ-10102A
- Deionized water or double-distilled water (ddH_2O)
- Coplin plastic staining jar, **Thermo Fisher Catalog #** 107
- Pipette tips, 1000 µL, **Fisher Scientific Catalog #** 21-403-03
- Pipette tips, 200 µL, **Fisher Scientific Catalog #** 21-236-1
- Pipette tips, 100 µL, **Fisher Scientific Catalog #** 212368
- Pipette tips, 10 µL, **Fisher Scientific Catalog #** 21-236-3
- Lint-free blotting cloth, **Fisher Scientific Catalog #** 50-192-9113
- Plastic staining rack (24 slides), **Fisher Scientific Catalog #** 22-445-668
- Plastic staining container (24 slides), **Fisher Scientific Catalog #** 22-445-844
- Slide humidity chamber (opaque black plastic; 10 slide), **Fisher Scientific Catalog #** NC9062083
- Slide bleaching chamber, **Fisher Scientific Catalog #** 12-600-004
- LED light source for slide bleaching, **Verilux Happylight Catalog #** VT41
- LED light source for slide bleaching, **GAGNE Catalog #** 675

Safety warnings

Handle all reagents with appropriate PPE. Consult with the MSDS website for more specific information on chemicals in the protocol.

- Ethanol is a highly flammable liquid and vapor. Keep away from heat and flames, use in a well-ventilated area, and avoid inhalation or prolonged skin contact. Wear appropriate PPE when working with ethanol.
- 5M NaOH is a highly corrosive base that can cause severe burns to skin and eyes. Handle with appropriate PPE, including gloves, safety goggles, and a lab coat. Use in a well-ventilated area and avoid inhalation or skin contact.
- Histo-Clear II is a flammable liquid and may cause skin and eye irritation. Keep away from heat and flames and use in a well-ventilated area. Wear appropriate PPE when working with Histo-Clear II.
- 30% hydrogen peroxide is a strong oxidizer and can cause severe skin and eye burns. Handle with appropriate PPE and avoid contact with skin, eyes, and clothing.
- ImmEdge Barrier PEN (PAP pen) contains organic solvents that may be harmful if inhaled or absorbed through the skin. Use in a well-ventilated area and avoid prolonged exposure.
- Prolong Gold Mounting Media with DAPI contains DAPI, a potential mutagen. Avoid skin and eye contact and do not ingest. Wear appropriate PPE and dispose of properly according to institutional guidelines.

Slide Preparation

1h 57m

1 Collect fresh 4% PFA-fixed paraffin-embedded (PFA-PE) or formalin-fixed PE (FFPE) sections (4 µm thickness) on Superfrost Plus microscope slides. Air dry slides overnight at room temperature and store until use.

2 Heat slides for 00:30:00 at 60 °C on a warming plate.

- **CRITICAL STEP** This heat treatment helps the tissue section to dewax and better adhere to the slide through multiple antigen retrieval steps.

3 Immediately dewax slides by placing them in a slide rack and incubating sequentially in slide containers with the following solutions:

3.1 00:05:00 in Histo-Clear, 00:05:00 in Histo-Clear, 00:05:00 in 100% ethanol, 00:05:00 in 100% ethanol, 00:05:00 in 95% ethanol, and 00:05:00 in 70% ethanol. 30m

CRITICAL STEP Do not permit tissue sections to dry out at any point after dewaxing starts. Incubation steps are performed at room temperature except where noted.

3.2 Wash slides for 00:05:00 in 1X tris-buffered saline with Tween 20 (1X TBST). 5m

Antigen Retrieval

1h 5m

4 Perform first antigen retrieval.

4.1 Make 1X AR9 solution using 10X stock diluted with ddH_2O by adding 1 mL 10X AR9 stock to 9 mL ddH_2O. Scale up volume as needed for all antigen retrieval steps in each cycle.

4.2 Preheat 1X AR9 solution in 200 mL staining container in steamer to 95 °C for 15 minutes or until the solution reaches temperature. Alternative heating devices can include water bath, pressure cooker, or microwave following validation with antibodies of interest.

4.3 Transfer slides from the 1X TBST into preheated 1X AR9 solution and incubate for 00:25:00 in the steamer. 25m

4.4 Remove the slide staining container with 1X AR9 solution and place on countertop to cool for 00:30:00. Use tongs or mitts to avoid burning one hands. 30m

- **CRITICAL STEP** For subsequent antigen retrieval steps, slides should be kept in the dark during all steps by using opaque staining containers and slide humidity chambers and avoid excessive exposure to light.

4.5 Wash slides 2 times for 00:05:00 each in deionized water. 5m

4.6 Wash slides once for 00:05:00 in 1X TBST. 5m

Optional Step: Create Hydrophobic Barrier around Tissue 1h 57m

5 Remove slides from slide container one by one and gently wipe around the tissue with a lint-free cloth (cut into 1×2 inch squares for easier use). **Optional:** Draw two vertical lines on either side of the tissue section with a wax PAP pen to create a hydrophobic barrier for incubation solutions.

Multiplex Immunofluorescence (MxIF) 1h 57m

6 Incubate sections with primary and secondary antibodies.

- **CRITICAL STEP** Design stain cycles to avoid cross-reactivity between antibodies from the same host species. The protocol starts with sequential application of primary antibodies using Opal detection followed by a cocktail of primary antibodies that are detected with a cocktail of immunofluorescence-labeled secondary antibody for different host species.
- **CRITICAL STEP** Since the Opal reagent protocol ends with antigen retrieval to strip off primary and secondary antibodies, all standard immunofluorescence primary/secondary antibody combinations must be done after the last Opal incubation.
- **CRITICAL STEP** Antibody incubations may be performed for 16 hours at 4 °C refrigeration depending on optimal conditions for each antibody or workflow.

Opal Reagents 1h 45m

7 Perform first protein block.

7.1 Remove slide(s) from 1X TBST wash buffer, gently blot off excess liquid around the section with lint-free cloth (cut into 1×2 inch squares for easier use) and place in humidity chamber.

- **CRITICAL STEP** All protein blocks, antibody incubations, and Opal reagent incubation steps are performed in a humidity chamber to avoid drying out.

7.2 Add 4 drops (200 µL) of blocking solution (Opal Antibody Diluent/Block) to each slide.

- **CRITICAL STEP** Repeat steps 7.1-7.2 for all slides in the set. Add solution to 1-4 slides at a time to prevent the tissue from drying out.

7.3 Incubate in protein block for 00:10:00 . 10m

8 Blot off excess protein block solution from slides and incubate with first primary antibody.

8.1 Dilute primary antibodies using Opal Antibody Diluent/Block.

8.2 One by one, remove slides from humidity chamber and drain off protein block solution by turning the slide on its side and gently tapping excess solution onto lint-free cloth. Then add primary antibody solution and place back in humidity chamber.

8.3 Use 200 µL primary antibody solution per slide.

8.4 Incubate for 01:00:00 . 1h

8.5 Wash 3 times for 00:05:00 each in 1X TBST. 5m

9 Incubate sections with Opal-compatible secondary antibody.

- For mouse/rabbit primaries, use 1X Opal Anti-Ms + Rb IgG HRP.
- For goat/sheep primaries, use HRP Horse Anti-Goat IgG Polymer Reagent.
- For rat primaries, use HRP Goat Anti-Rat IgG Polymer Reagent.

9.1 Use 200 µL secondary antibody solution per slide.

9.2 Incubate for 00:10:00 . 10m

9.3 Wash 3 times for 00:05:00 each in 1X TBST. 5m

10 Incubate slides with selected Opal reagent.
- **CRITICAL STEP** Test different Opal fluorophores to determine which ones work best with a given primary antibody. In our testing, Opal 520, Opal 570, Opal 650, and Opal 780 worked well for multiple antibodies and could be bleached under these conditions.
- **CRITICAL STEP** Reconstitute stock Opal fluorophores with DMSO according to manufacturer's manual. Dilute Opal fluorophores in 1X Amplification Diluent (1:100 dilution).

10.1 Use 200 µL Opal solution per slide.

10.2 Incubate for 00:10:00 . 10m

10.3 Wash 3 times for 00:05:00 each in 1X TBST. 5m

11 Perform next antigen retrieval according to step 4 of the protocol.

12 Repeat steps 7-11 for any remaining primary antibodies using different Opal fluorophores.

Traditional Immunofluorescence Reagents 3h 40m

13 Perform protein block according to step 7 and incubate for 00:30:00 instead of 10 minutes. 30m

14 Apply primary antibody cocktail.

14.1 Drain protein block solution from slides and incubate with first primary antibody.
- **CRITICAL STEP** Multiple primary antibodies from different host species are incubated simultaneously in a single primary antibody cocktail.

14.2 Dilute primary antibodies using Opal Antibody Diluent/Block.

14.3 One by one, remove slides from humidity chamber and drain off protein block solution by turning the slide on its side and gently tapping excess solution onto lint-free cloth. Then add primary antibody solution and place back in humidity chamber.

14.4 Use 200 µL primary antibody solution per slide. 1h

14.5 Incubate for 01:00:00 . 1h

14.6 Wash 3 times for 00:05:00 each in 1X TBST. 5m

15 Incubate slides with secondary antibodies.

15.1 Dilute secondary antibodies using Opal Antibody Diluent/Block using 200 µL secondary antibody solution per slide .

15.2 Incubate for 01:00:00 . 1h

15.3 Wash 3 times for 00:05:00 each in 1X TBST. 5m

Nuclear Counterstain and Coverslip 17m

16 Perform nuclear counterstain. 17m

- **CRITICAL STEP** Nuclear counterstain must be completed in each cycle to facilitate co-registration of cycle images and downstream image analysis.

16.1 Option A: Hoechst S769121 (nuclear yellow): Dilute stock nuclear yellow 1:50 in 1X TBST and incubate slides for 00:15:00 . Diluted nuclear yellow can also be added to the secondary antibody cocktail and incubated as indicated.
Following incubation, wash 3 times for 00:05:00 each in 1X TBST. Mount using aqueous Fluoromount-G anti-fade mounting media (30-70 µL per slide).

- **NOTE** Hoechst S769121 (nuclear yellow) is maximally excited at 360 nm with a long Stokes shift at 505 nm. Nuclear yellow can be used with other UV excitable fluorophores such as Alexa fluor 405, BV421, and Opal 480 by using suitable filter cubes or confocal microscopes to separate emission spectra of the UV fluorophores from nuclear yellow. Long pass DAPI filters will not sufficiently separate nuclear yellow signal from other UV fluorophores. Obtain appropriate filters or image using a confocal microscope to detect separate emission spectra.

16.2 Option B: Use Prolong Gold Mounting Media with DAPI (30-70 µL per slide). Image using a DAPI filter.

17 Apply 22×50mm No. 1.5 coverslips. Gently press down on the coverslip to remove any air bubbles, wipe off excess media from edges, and air dry in dark before imaging.

MxIF Imaging

34m

18 Following each cycle, photograph slides using a fluorescence microscope or slide scanner with appropriate filters for each fluorophore.

18.1 Co-register staining cycle images using ImageJ's QuPATH Warpy extension (https://imagej.net/plugins/bdv/warpy/warpy-extension).

18.2 Analyze using QuPATH software (**https://qupath.github.io/**).

Strip Antibodies and Bleach Slides

1h 5m

19 Soak slides vertically in 1X PBS buffer until coverslips come off.

- **CRITICAL STEP** This can take anywhere from 1 hour to 24 hours depending on how long since slides were stained and cover-slipped.

20 Perform antigen retrieval according to step 4 of the protocol to strip primary and secondary antibodies from tissue.

21 Prepare slide bleaching solution on ice using ice cold reagents.

21.1 Prepare 10 mL of bleaching solution per petri dish (scale up accordingly).

- **CRITICAL STEP** Bleaching solution is only active for a short amount of time so prepare right before use.

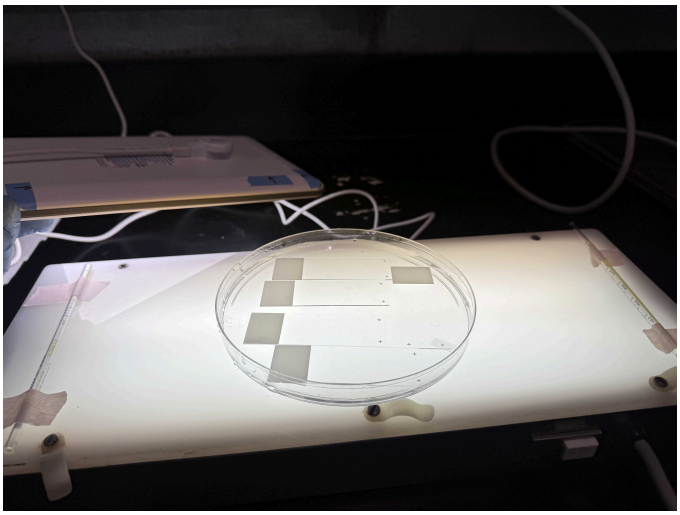
21.2 Bleaching reagents (add in order listed):

- 7.96 mL ddH$_2$O
- 1 mL 10X PBS
- 40 µL 5M NaOH
- 1 mL 30% H_2O_2 – add last, right before using.

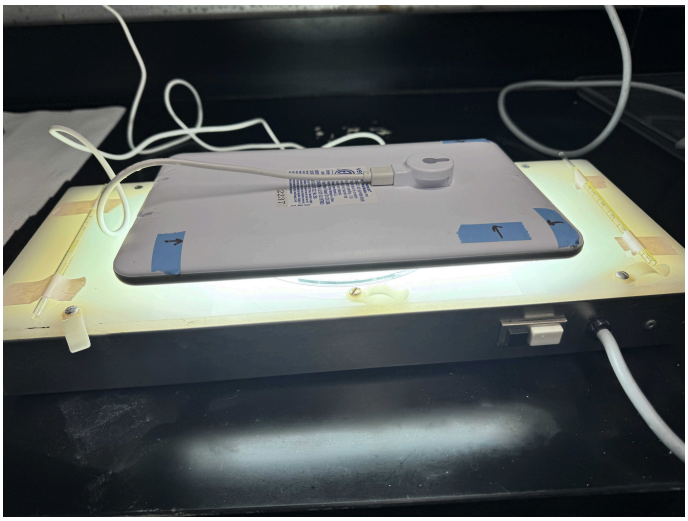
21.3 Add slide bleaching solution to petri dish or other bleaching container.

22 Take slides out of 1X PBS, tap off excess PBS, and place face up in bleaching solution.

23 Place petri dish with slides on top of LED light source at 4 °C. Place another LED light source on top of the petri dish with lid off to bleach from both sides.



Slide bleaching container (petri dish) on top of LED light source.



Slide bleaching container sandwiched between two LED light sources.

23.1 Incubate for 00:30:00 at 4 °C . 30m

- **CRITICAL STEP** We use a cold room for bleaching procedures but an ice tray can be used if a cold room is not available.

24 Prepare fresh bleaching solution according to step 21. Discard old bleaching solution and add fresh solution to petri dish with slides.

24.1 Incubate for another 00:30:00 at 4 °C . 30m

- **CRITICAL STEP** Check slides with fluorescent microscope to confirm bleaching. Continue rounds of bleaching until slides have sufficiently lost fluorescence. Three rounds of bleaching will generally achieve adequate bleaching with these fluorophores.

25 Wash slides 3 times for 00:05:00 each in 1X PBS. Store slides in 1X PBS at 4 °C until next staining cycle. 5m

26 Return to step 4 of the protocol and repeat for additional cycles.

Protocol references

Publications:
Lin JR, Izar B, Wang S, Yapp C, Mei S, Shah PM, Santagata S, Sorger PK. Highly multiplexed immunofluorescence imaging of human tissues and tumors using t-CyCIF and conventional optical microscopes. *eLife*. 2018;7:e31657. doi:10.7554/eLife.31657

Bady P, Krayem M, Valge-Archer V, et al. Bleach & stain protocol for high-sensitivity multiplexed immunofluorescence imaging of archival tissue sections. *Nat Biotechnol.* 2023. doi:10.1038/s41587-023-01878-w

Protocols:
Human Pancreas Processing (**dx.doi.org/10.17504/protocols.io.n2bvj6dnblk5/v1**)
Tissue Cyclic Immunofluorescence (t-CyCIF) (**dx.doi.org/10.17504/protocols.io.bjiukkew**)

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