Immunofluorescence Multi-label Protocol for Free-floating Fixed Tissue

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

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ABSTRACT

Immunofluorescence multi-label protocol for staining free-floating fixed tissue in the Kordower Laboratory.

ATTACHMENTS

Immunofluor Multi-Label with Same Species Staining Protocol.docx

GUIDELINES

HISTO- NOTES:

- Primate tissue staining dishes use 100 mL solution per dish
- Rodent tissue staining dishes 50 mL solution per dish
- If staining a large number of primate cases, incubate 1' & 2' Ab in individual cups to conserve volume of Ab used.
- Be conscious of tissue saturation while washing and incubating. i.e. Check that tissue is fully submerged in solution & not clumping. This will ensure proper penetration of antibodies & other reagents.
- Always include Positive & Negative Controls.
- Positive: Use relevant control tissue to confirm specific antibody detection. (i.e. pS129; control tissue should consist of nigral sections previously successfully stained for pS129).
- Negative: Ideally, use tissue that you know does not contain the targeted antigen. If not available, use a section of tissue not incubated in the 1' Ab (primary delete).
- When incubating 1' Ab overnight, leave on shaker in refrigerator.
- Can incubate in fridge on a shaker, covered in parafilm, over the weekend or up to 3 days.
- Select a secondary antibody directed against the species in which the primary antibody was raised (i.e. if a primary antibody raised in rabbit is used, an anti-rabbit secondary antibody raised in a species other than rabbit must be used).

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MATERIALS

- Dilution Media (DM) ([M] 0.2 Molarity (M) TBS plus [M] 0.05 % volume Triton X-100)
- [M] 0.2 Molarity (m) Tris-buffered saline (TBS)
- Sodium meta-periodate
- Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- Bovine Serum Albumin (BSA)
- Triton X-100
- Vectastain Elite ABC-HRP Kit (PK-6100)
- Imidazole
- Sodium Acetate
- 3,3-Diaminobenzidine Tetrahydrochloride (DAB)
- [M] 30 % (v/v) hydrogen peroxide
- [M] 0.2 Molarity (m) Phosphate-buffered saline (PBS)
- Household Bleach
- Primary antibody against the target antigen
- Secondary antibody directed against the species in which the primary antibody was raised (i.e. if a primary antibody raised in rabbit is used, an anti-rabbit secondary antibody raised in a species other than rabbit must be used).

DAY 1 (4 hrs)

1 Wash sections (6 x 00:10:00) in Dilution Media (DM) ([M] 0.2 Molarity (M) TBS plus [M] 0.05 % volume Triton X-100). 10m

1.1 Wash sections for 00:10:00 in DM (1/6). 10m

1.2 Wash sections for 00:10:00 in DM (2/6). 10m

1.3 Wash sections for 00:10:00 in DM (3/6). 10m

1.4 Wash sections for 00:10:00 in DM (4/6). 10m

1.5 Wash sections for 00:10:00 in DM (5/6). 10m

1.6 Wash sections for 00:10:00 in DM (6/6). 10m

2 Endogenous peroxidase inhibition (00:20:00). [M] 0.1 Molarity (M) Sodium meta-periodate in TBS. 20m

> **Note**
>
> **Only necessary if using ABC, because it has HRP and could cause background.**

- 100 mL [M] 0.2 Molarity (m) Tris-buffered saline (TBS)
- 2.13 g Sodium meta-periodate

3 Wash (2 x 00:10:00) in DM. 10m

> **Note**
>
> **Only necessary following endogenous peroxidase inhibition.**

3.1 Wash for 00:10:00 in DM (1/2). 10m

3.2 Wash for 00:10:00 in DM (2/2). 10m

4 Serum blocking step (01:00:00 incubation): 1h

- 100 mL DM
- 3 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 2 g Bovine Serum Albumin (BSA)

5 Incubation in primary antibody (18:00:00 - 72:00:00). See antibody catalog for concentra 3d 18h
of primary antibody.

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA
- 0.5 mL Triton X-100

Note

Optionally, refrigerate 4 °C to keep antibody stable

DAY 2 (8 hrs) 2h 30m

6 Wash (6 x 00:10:00) in DM. 10m

6.1 Wash in DM for 00:10:00 (1/6). 10m

6.2 Wash in DM for 00:10:00 (2/6). 10m

6.3 Wash in DM for 00:10:00 (3/6). 10m

6.4 Wash in DM for 00:10:00 (4/6). 10m

6.5 Wash in DM for 00:10:00 (5/6). 10m

6.6 Wash in DM for 00:10:00 (6/6). 10m

7 Fluorophore-conjugated secondary antibody incubation. (01:00:00) Concentration of secondary antibody is always 1:200 in solvent. 1h

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA

8 Wash (2 x 00:10:00) in TBS. 10m

8.1 Wash for 00:10:00 in TBS (1/2). 10m

8.2 Wash for 00:10:00 in TBS (2/2). 10m

9 Serum blocking step (01:00:00 incubation):

- 100 mL DM
- [M] 5 % (v/v) Normal Serum (species **MATCHING THE HOST OF THE PRIMARY** antibody, saturates open binding sites on secondary antibody)

- 1 g Bovine Serum Albumin (BSA)

Note

****DO NOT USE any detergent (i.e. Triton X-100, Tween-20, DM) from this step onward! Detergent will wash away the fragment antibodies!****

10 Oversaturate with Fab antibody **against host of primary antibody** and from **same host species as secondary antibody**. (ex. If primary was mouse and secondary was goat anti-mouse, you would use a Fab-goat **anti-mouse** antibody). Working concentration: [M] 40 µg/mL . (01:00:00 incubation) 1h

- 100 mL TBS
- [M] 40 µg/mL Fab antibody (base concentration is 1.3 mg/mL for fab-goat anti mouse, so use M1V1 = M2V2 to find V1/X).

11 Wash (2 x 00:10:00) in TBS.

11.1 Wash for 00:10:00 in TBS (1/2).

11.2 Wash for 00:10:00 in TBS (2/2).

12 Incubation in **SECOND** primary antibody (18:00:00 - 72:00:00). See antibody catalog for concentration of primary antibody.

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA

Note

****Optionally, refrigerate 4 °C to keep antibody stable** CAN ALSO ADD THIRD PRIMARY ANTIBODY FROM DIFFERENT SPECIES, IF NEEDED.**

DAY 3 (2 hrs)

13 Wash (6 x 00:10:00) in TBS.

13.1 Wash in TBS for 00:10:00 (1/6).

13.2 Wash in TBS for 00:10:00 (2/6).

13.3 Wash in TBS for 00:10:00 (3/6).

13.4 Wash in TBS for 00:10:00 (4/6).

13.5 Wash in TBS for 00:10:00 (5/6).

13.6 Wash in TBS for 00:10:00 (6/6).

14 Fluorophore-conjugated secondary antibody incubation against second (and third, if used) primary antibody. (01:00:00) Use different fluorophores for each secondary. Concentration of secondary antibody is always 1:200 in solvent.

- 100 mL DM
- 1 mL Normal Serum (species matching the host of the secondary antibody, e.g. horse, goat)
- 1 g BSA

15 Wash (3 x 00:10:00) in TBS.

15.1 Wash for 00:10:00 in TBS (1/3).

15.2 Wash for 00:10:00 in TBS (2/3).

Note

****Can add DAPI (1:15,000) during the second TBS washing step, if desired.****

15.3 Wash for 00:10:00 in TBS (3/3).

16 Store tissue in TBS in the refrigerator at 4 °C until mounted.

17 Control for Fragment antibody (Fab): Control tissue should be processed alongside experimental tissue through Day 2, Step 11. Skip second primary incubation all together (Step 12), and complete Day 3. Check under microscope to ensure there is no co-labeling between the two chosen fluorophores.
Use appropriate +/- controls.