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Labeling of microtubules using mouse anti-β-tubulin primary monoclonal antibody with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate and oxidation of DAB with H2O2 for light and transmission electron microscopy

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We use this protocol and it's working.

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Abstract

This protocol details the labeling of microtubules using mouse anti-β-tubulin primary monoclonal antibody with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate and oxidation of DAB with H_2O_2 for light and transmission electron microscopy.

Materials

CSB buffer:

	A	B
	Pipes buffer	10 mM
	NaCl	150 mM
	EGTA	5 mM
	MgCl2	5 mM
	Glucose monohydrate, pH 6.8	5 mM

- Anti-β-Tubulin antibody, Mouse monoclonal, clone TUB 2.1 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**T5201
- 3,3′-Diaminobenzidine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D8001- 10G
- Durcupan™ ACM **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**44610
- Fe-TAML-peg4-Cy5-goat anti-mouse IgG
- BSA **Sigma Catalog #A8022-100G**

Labeling of microtubules

2d 17h 59m

1 Culture HEK293T cells on MatTek plates containing poly-D-lysine coated glass bottom No.0 coverslips in DMEM supplement with 10% fetal bovine serum.

2 Rinse the cells (x3) with cytoskeleton stabilizing buffer, at 37 °C and fixed with 4% paraformaldehyde (19202, Electron Microscopy Sciences) and 0.05% glutaraldehyde (16220, Electron Microscopy Sciences) in CSB at 37 °C for 00:05:00 and for 00:25:00 at 4 °C. 30m

CSB buffer:

	A	B
	Pipes buffer	10 mM
	NaCl	150 mM
	EGTA	5 mM
	MgCl2	5 mM
	Glucose monohydrate, pH 6.8	5 mM

3 After fixation, first wash the cells with CSB (5 x 1 min) at 4 °C.

3.1 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (1/5) 1m

3.2 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (2/5) 1m

3.3 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (3/5) 1m

3.4 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (4/5) 1m

3.5 After fixation, first wash the cells with CSB for 00:01:00 at 4 °C (5/5) 1m

4 Treat with 0.1% saponin and 0.05% glycine in CSB for 20 mins at 4 °C while on a rocker.

5 Wash the cells in CSB buffer with 0.05% glycine (3 x 1 min) at 4°C.

5.1 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (1/3) 1m

5.2 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (2/3) 1m

5.3 Wash the cells in CSB buffer with 0.05% glycine 00:01:00 at 4 °C. (3/3) 1m

6 Block the cells with 1% BSA (A8022-100G, Sigma), 1% normal goat serum (NGS) and 0.05% glycine in CSB for 00:20:00 at 4 °C. 20m

7 Incubate the cells with primary mouse monoclonal antibody to β-tubulin (300-fold dilution, clone Tub2.1, T5201, Sigma) for 03:00:00 at 4 °C in 1% BSA, 1% NGS and 0.05% glycine in CSB buffer. 3h

8 Remove primary antibody and wash with 1% BSA, 1% NGS and 0.05% glycine in CSB (5 x 3 min) at 4°C.

8.1 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (1/5) 3m

8.2 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (2/5) 3m

8.3 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (3/5) 3m

8.4 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (4/5) 3m

8.5 Wash with 1% BSA, 1% NGS and 0.05% glycine in CSB 00:03:00 at 4 °C. (5/5) 3m

9 Incubate the cells with secondary Fe-TAML-peg4-Cy5-goat anti-mouse IgG conjugate in 1% BSA (0.15 ml diluted to 1ml) in 1% NGS and 0.05% glycine in CSB for Overnight at 4 °C. [Adams et al., 2023]. 8h

10 Then wash the cells (5 x 1 min) with CSB at 4°C.

10.1 Wash the cells for 00:01:00 with CSB at 4 °C. (1/5) 1m

10.2 Wash the cells for 00:01:00 with CSB at 4 °C. (2/5) 1m

10.3 Wash the cells for 00:01:00 with CSB at 4 °C. (3/5) 1m

10.4 Wash the cells for 00:01:00 with CSB at 4 °C. (4/5) 1m

10.5 Wash the cells for 00:01:00 with CSB at 4 °C. (5/5) 1m

11 Fix the cells with 2% glutaraldehyde in CSB for 00:20:00 at 4 °C. 20m

12 Wash the cells (5 x 1 min) with CSB at 4°C.

12.1 Wash the cells for 00:01:00 with CSB at 4 °C . (1/5) 1m

12.2 Wash the cells for 00:01:00 with CSB at 4 °C . (2/5) 1m

12.3 Wash the cells for 00:01:00 with CSB at 4 °C . (3/5) 1m

12.4 Wash the cells for 00:01:00 with CSB at 4 °C . (4/5) 1m

12.5 Wash the cells for 00:01:00 with CSB at 4 °C . (5/5) 1m

13 Image the cells for fluorescence labeling.

14 Dissolve and add 5.4 mg of 3,3'- Diaminobenzidine (DAB) (D8001-10G, Sigma-Aldrich) in 1.0 mL of [M] 0.1 Mass Percent HCl and 9.0 mL of [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 with 10 µL H_2O_2 (final, [M] 40 millimolar (mM) from 30% stock) to the DAB solution.

15 Wash the cells (2 x 2 min) with 50 mM Bicine 100 mM NaCl pH 8.3 at 4°C.

15.1 Wash the cells for 00:02:00 with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 at 4 °C . (1/2) 2m

15.2 Wash the cells for 00:02:00 with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 at 4 °C . (2/2) 2m

16 Add the DAB/H_2O_2 solution to the cells by a 0.22µm Millex 33mm PES sterile filter (SLGSR33RS, Sigma-Aldrich) at Room temperature . Reaction time is 01:00:00 - 1h 30m

01:30:00 .

17 Remove the DAB solution, and wash the cells with 50 mM Bicine 100mM NaCl pH 8.3 (2 x 2 min) on ice.

17.1 Wash the cells with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 for 00:02:00 On ice . (1/2) 2m

17.2 Wash the cells with [M] 50 millimolar (mM) Bicine [M] 100 millimolar (mM) NaCl pH 8.3 for 00:02:00 On ice . (2/2) 2m

18 Wash the cells with 0.1 M sodium cacodylate buffer pH 7.4 (3 x 2 min) on ice.

18.1 Wash the cells with [M] 0.1 Molarity (M) sodium cacodylate buffer pH 7.4 for 00:02:00 . (1/3) 2m

18.2 Wash the cells with [M] 0.1 Molarity (M) sodium cacodylate buffer pH 7.4 for 00:02:00 . (2/3) 2m

18.3 Wash the cells with [M] 0.1 Molarity (M) sodium cacodylate buffer pH 7.4 for 00:02:00 . (3/3) 2m

19 Do a final primary fixation with 2% glutaraldehyde in [M] 2 millimolar (mM) $CaCl_2$ [M] 0.1 Molarity (M) sodium cacodylate pH 7.4 for 00:30:00 at 4 °C . 30m

20 Remove the fixative and wash the cells (5 x 2 min) with 0.1 M sodium cacodylate (18851, Ted Pella) pH 7.4 at 4°C.

20.1 Wash the cells for 00:02:00 with [M] 0.1 Molarity (M) sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C . (1/5) 2m

20.2 Wash the cells for 00:02:00 with [M] 0.1 Molarity (M) sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (2/5) 2m

20.3 Wash the cells for 00:02:00 with [M] 0.1 Molarity (M) sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (3/5) 2m

20.4 Wash the cells for 00:02:00 with [M] 0.1 Molarity (M) sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (4/5) 2m

20.5 Wash the cells for 00:02:00 with [M] 0.1 Molarity (M) sodium cacodylate (18851, Ted Pella) pH 7.4 at 4 °C. (5/5) 2m

21 All cells are post-fixed with 1% osmium tetroxide (19150, Electron Microscopy Sciences) containing 0.8% potassium ferrocyanide, [M] 2 millimolar (mM) $CaCl_2$ and in [M] 0.1 Molarity (M) sodium cacodylate pH 7.4 for 00:30:00 at 4 °C. 30m

22 Wash the cells (5 x 2 min) with ddH_2O at 4°C.

22.1 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (1/5) 2m

22.2 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (2/5) 2m

22.3 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (3/5) 2m

22.4 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (4/5) 2m

22.5 Wash the cells for 00:02:00 with ddH_2O at 4 °C. (5/5) 2m

23 Dehydrate the cells by an ice-cold graded dehydration ethanol series of 20%, 50%, 70%, 90%, 100% (anhydrous) for 00:01:00 each and 3 x 100% (anhydrous) at Room temperature for 00:01:00 each. 2m

24 Infiltrate the cells with one-part Durcupan ACM epoxy resin (44610, Sigma-Aldrich) to one-part anhydrous ethanol (1:1) for 00:30:00 . 30m

25 Infiltrate the cells 3 times with 100% Durcupan resin for 01:00:00 each. 1h

26 Do a final change of Durcupan resin and immediately place cells in a vacuum oven at 60 °C for 48:00:00 to harden. 2d

Protocol references

Adams, Stephen R., et al. "Fe-TAMLs as a new class of small molecule peroxidase probes for correlated light and electron microscopy." *bioRxiv* (2023): 2023-08.