A Modified Approach of Fluoro-Jade C Labeling for Neurotoxicity Assessments

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Abstract

Fluoro-Jade C (FJ-C) stain is a histochemical method for the labeling of degenerating neurons and has been widely used for the detection and confirmation of neurotoxicity in the central nervous system. In this study, we present a modified approach of FJ-C labeling in animals treated with kainic acid (KA) to induce neurotoxicity. The modified approach requires fewer chemical reagents and incubation steps than the conventional FJ-C labeling method. This modified approach also allows co-staining with other fluorescence-based histochemical and/or immunohistochemical methods simultaneously in the same tissue section for double- or multi-color labeling. FJ-C-labeled cells could be imaged regardless of whether tissue sections were wet or dried and cover-slipped, thus providing more flexibility if needed. More importantly, the modified approach demonstrated higher sensitivity than the conventional FJ-C labeling method. For example, when animals received KA-treatment but did not develop severe seizures conventional FJ-C stain failed to display labeled neurons in those brains. However adjacent brain tissue sections of the same KA-treated animals could still show FJ-C positive neurons when using the modified approach, suggesting neurotoxicity indeed occurred in those KA-treated animals that had mild seizures. In conclusion, this modified FJ-C labeling approach could be utilized as an added method for neurotoxicity assessments.

Introduction

Fluoro-Jade C (FJ-C) is a fluorescent tracer derived from fluorescein and has been widely used for histochemical labeling of degenerating neurons. Compared to its two predecessors, Fluoro-Jade and Fluoro-Jade B, FJ-C results in tissue staining with higher resolution, higher contrast, and lower background noise. FJ-C labeling has been applied in various studies such as traumatic brain injury, developmental apoptosis, ischemia, and animal models of neurodegenerative diseases and assessing neurotoxicity. The FJ-C stain is among a few techniques commonly used for neurotoxicity assessment. Beside finding degenerating neurons as histopathological evidence of neurotoxicity, contemporary neurotoxicity assessments often want co-localization analyses of degenerating neurons with specific cellular and/or molecular markers to better understand signaling pathways and mechanisms underlying neurotoxicity. Since the conventional FJ-C staining method uses potassium-permanganate, which makes tissue sections dark-brown (Figure 1), it is difficult if not impossible to conduct double or multiple labeling of FJ-C with immunocytochemical labeling of other cellular or molecular targets in the same tissue section. This presentation introduces a modified FJ-C labeling approach, which avoids the chemical potassium permanganate. As a proof-of-concept, a triple labeling of degenerating neurons (FJ-C), GABAergic neurons (GAD), and nuclei (DAPI) in the same tissue section is demonstrated. Also, this new approach needs less chemicals and omits using other chemicals employed for the conventional FJ-C staining method such as the basic alcohol, ethanol, and acetic acid, and shows a higher sensitivity to identify degenerating neurons following neurotoxicant insults than the conventional FJ-C staining method.

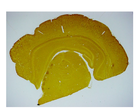

Figure 1. A rat brain section was processed with the traditional FJ-C labeling method. Note the dark-brown color after the staining procedure.

Methods

Animals and treatments:

Adult male Sprague Dawley rats and 6-week-old male C57BL/6J mice were used in this study. To generate neurotoxicity in the brain, animals were treated with intraperitoneal (*i.p.*) injection of kainic acid (KA, 10 mg/kg bodyweight of rats or 30 mg/kg bodyweight of mice). Animals receiving *i.p.* injection of saline (same volume as calculated for KA) were used as controls. Twenty-four hours after KA- or saline-injection, animals were euthanized by *i.p.* injection of pentobarbital (100 mg/kg bodyweight), perfused with phosphate buffer saline (PBS, 0.1 M, pH 7.4) and followed by 4% paraformaldehyde (PFA) for 5 minutes. After perfusion, brain samples were collected and post-fixed in PFA at 4°C until use. Fixed brain samples were submerged in 30% sucrose overnight and sectioned using a cryostat at 25 μm.

FJ-C labeling procedures:

Conventional FJ-C stain and the modified approach are shown in Figure 2.

Traditional FJ-C protocol	New FJ-C approach
- Wash tissue sections in 1x PBS	- Wash tissue sections in 0.1x PBS
- Mount on glass slide	- Incubate in 0.1x PBS containing 0.0001% FJ-C overnight
- Air-dry on a slide warmer	- Wash in 0.1x PBS
- 5 min in basic alcohol	- Mount on glass slide
- 2 min in 70% ethanol	- Air-dry
- 2 min in dH_2O	- Dehydration
- 10 min in 0.06% $KMnO_4$	- Coverslip
- 2 min in dH_2O	- Imaging
- 10 min in 0.1% acetic acid (pH = 3.2) containing 0.0001% FJ-C	
- 2 min in dH_2O	
- 2 min in dH_2O	
- Air-dry	
- Dehydration	
- Coverslip	
- Imaging	

Figure 2. Side-by-side comparison of the traditional and the new FJ-C labeling steps.

Triple labeling (GAD, FJ-C, and DAPI) procedures:

- Wash with PBS
- Incubation with 5% normal goat serum for 30 minutes
- Incubation with mouse monoclonal anti-glutamate-decarboxylase-67kDa-isoform (GAD67)-antibody (1:200) in antibody incubation medium (PBS containing 1% normal goat serum, 0.1% Kodak-Flo, and 0.00005% FJ-C) at 4°C overnight
- Wash with PBS
- Incubation with biotinylated goat-anti-mouse antibody (1:200) in antibody incubation medium for 2 hours
- Wash with PBS
- Incubation with Cy5-conjugated streptavidin (1:200) and DAPI (0.0001%) in antibody incubation medium for 30 minutes
- Wash with PBS
- Mount on glass slide
- Air-dry
- Dehydration
- Coverslip
- Imaging

Results

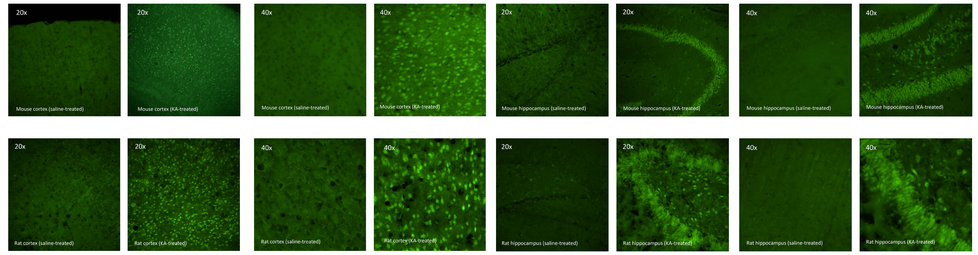

Figure 3. Mouse cortex and hippocampus sections following treatment with KA and saline, respectively, were processed using the new FJ-C labeling approach.

Figure 4. Rat cortex and hippocampus sections following treatment with KA and saline, respectively, were processed using the new FJ-C labeling approach.

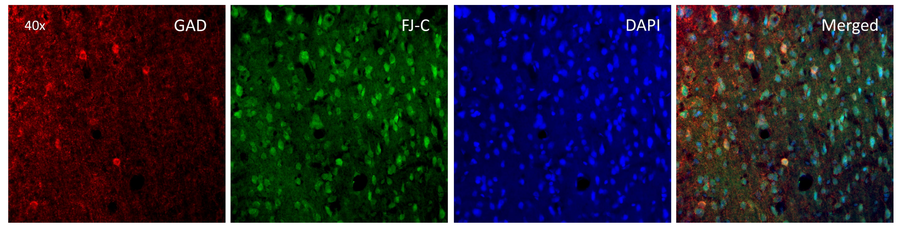

Figure 5. Triple labeling of GABAergic neurons (red), degenerating neurons (green), and nuclei (blue) in a cortex section of a rat that received KA-treatment. Arrows point to the same GABAergic neurons, degenerating neurons, and nuclei of these neurons in different panels.

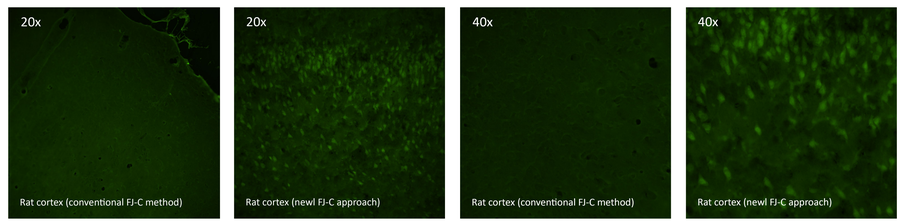

Figure 6. Two adjacent cortex sections of a rat that received KA-treatment but did not develop strong seizures. One tissue section was processed with the conventional FJ-C staining method while the other with the new FJ-C labeling approach.

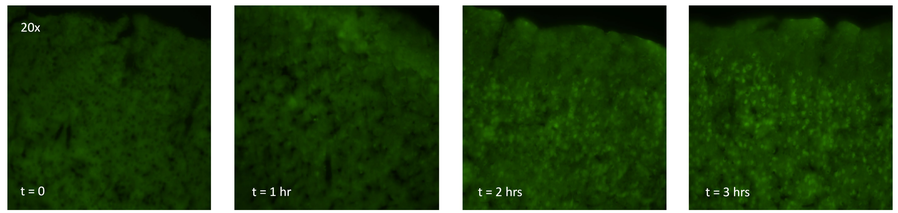

Figure 7. A cortical section of a mouse that received KA-treatment was processed with the new FJ-C labeling approach at different incubation times.

Acknowledgement

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Disclaimer

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Conclusion

The new FJ-C labeling approach has the following features compared with the conventional FJ-C labeling methods: 1) needs less chemicals and fewer processing steps, 2) enables multi-color labeling with other fluorescent markers, and 3) has a higher sensitivity to detect degenerating neurons. This new approach could be utilized as an added tool for neurotoxicity assessments and for co-localization analyses of multiple cellular and molecular targets combined with FJ-C labeled cells.