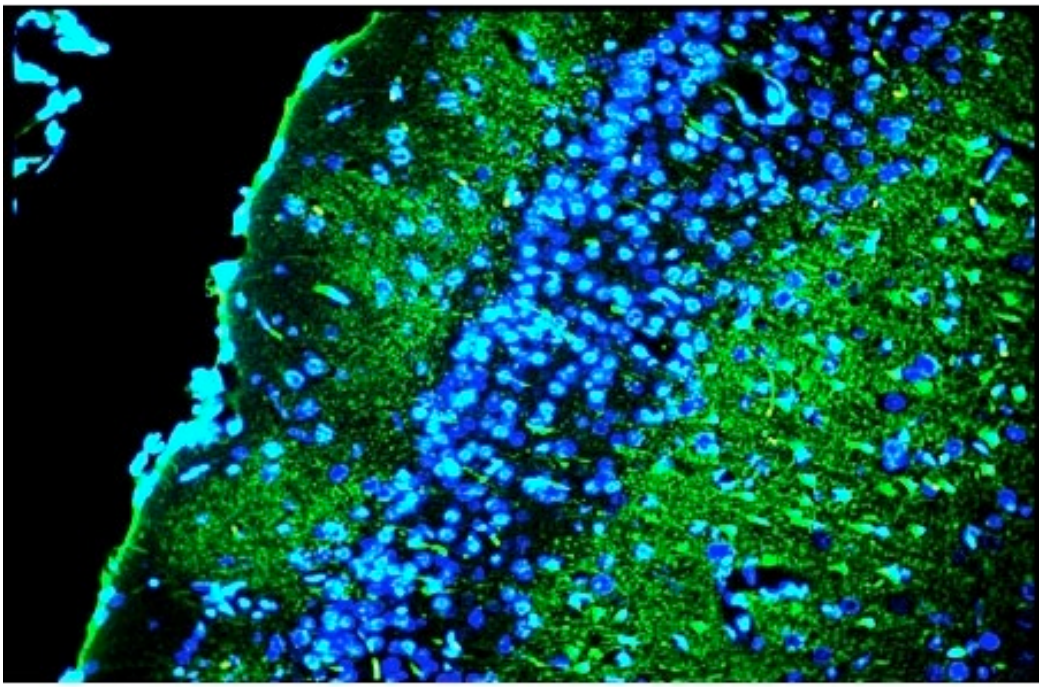
Fluoro-Jade® C POWDER Stain Reagent Protocol

Catalog Number: TR-160-FJC



For research use only, not for use in clinical and diagnostic procedures.

Description

The fluorescent dye contained in the Fluoro-Jade® C (FJC) staining kit stains for neuronal degeneration and can be used with other fluorescent Nissl stains or UV nuclear stain such as DAPI to help visualize the cells and cell nuclei. Our product TR-160-FJC contains only the powdered from of Fluoro- Jade® C (FJC). Full ready-to-dilute (RTD™) versions of FJC are available as TR-100-FJ or a trial version (TR-100-FJT). There is no trial powder version available. The FJC dye stain is compatible with all common fixations, embedding, sectioning and staining procedures, although the use of slide- mounted, formalin fixed, freeze-cut tissue sections is most typical. The powder is shipped at ambient temperature, see storage instructions for details.

Materials Provided

- 30 mg Fluoro-Jade® C dry powder
- Detailed protocol

Fluorescent Imaging Settings

Fluoro-Jade® C labeled degenerating neurons are visualized with blue light excitation (like those used for visualizing fluorescein or FITC), while DAPI counter stained cell nuclei are visualized with ultra-violet (UV) illumination. Fluoro-Jade® C has an excitation peak at 485- 506 nm and an emission peak at 525-527 nm. On a laser system, it can be excited using a 488 nm laser paired with a 515/20 nm bandpass filter.

Storage Conditions

The powdered dye can be stored desiccated at room temperature in the dark. Storage in a desiccator is recommended as FJC is hydroscopic. The 0.01% stock solution will remain stable for 3 months when stored in a refrigerator, in the dark. The 0.0001-0.0004% working solution in 0.1% acetic acid should be used within 4 hours of preparation. Diluted FJC dye solutions are not stable and should not be stored. The other diluted solutions can be reused and stored for up to 48 hours if refrigerated and protected from light. Best results require freshly diluted solutions.

We recommend using aseptic techniques when handling the reagents to avoid bacterial growth and contamination. The TR-160-FJC material is shipped ambient and stable at room temperature during transport.

Materials Required but not Supplied

- Gelatin-coated or tissue-treated microscope slides (i.e. Superfrost plus)
- Distilled water
- ACS grade Ethanol (200 proof) for slide & solution preparation
- 1% sodium hydroxide in 80% ethanol (basic alcohol solution).
- 0.1% Acetic Acid solution (in water)
- 70% ethanol in distilled water
- 0.06% (KMnO4) potassium permanganate solution
- DAPI powder or 100X solution (working range is 0.5-5 µg/mL)
- Xylene liquid
- Staining dishes/Coplin jars
- Cover slips
- DPX mounting media or another permanent mounting medium. Non-polar media are preferred over aqueous mounting media such as glycerin/water to obtain high- contrast images (refer to Appendix B for a comparative analysis). Traditional fluorescent mounting mediums are not recommended because of their high pH.
- Slide warmer
- Convection oven

Solution & Slide Preparations

Basic Alcohol solution:
Combine 20 mL of 5% NaOH in water with 80 mL absolute alcohol, mix and use immediately; solution can be stored at 4°C for up to a month.

Potassium permanganate ($KMnO_4$) 0.06% solution:
Make a 0.6% stock solution by adding 600 mg in to 100 mL water, filter, and store. Store 0.6% concentrate in the dark for up to 3 months. Just prior to use, dilute at a 9:1 ratio with distilled water to give a final potassium permanganate working solution concentration of 0.06%.

Fluoro-Jade® C 0.01% Stock Solution:
Dissolve 10 mg of Fluoro-Jade® C powder in 100 mL distilled water, filter through a 0.45 µm membrane and store at 4°C in the dark for up to 3 months. Discard if cloudy or precipitated.

Fluoro-Jade® C 0.0001-0.0004% Working solution:

Add 1 mL of the FJC 0.01% stock to 99 mL of solution of 0.1% acetic acid vehicle to make a 0.0001% working solution. Higher concentrations of working solutions can be achieved by adding more FJC stock solution, i.e. 0.0004% is achieved by adding 4 mL of 0.01% stock solution to 96 mL of 0.1% acetic acid solution. The working solution is not stable and needs to be used within 2 h of preparation and not stored.

Ethanol, 70% solution (500 mL):

In a graduated cylinder, add 7 parts absolute Ethanol to 3 parts distilled water, mix gently. Ethanol and water create an exothermic reaction so work slowly and carefully. Store in a sealed bottle at 4°C; remake after 3 months. For example adding 350 mL ethanol, then 150 mL water to 500 mL total volume provides a 70% ethanol solution.

Acetic Acid Solution 0.1% (500 mL):

The basic formula is 1 part of acetic acid and 499 part of water. For example, to prepare 500 mL of 0.1% acetic acid, dissolve 0.5 mL of glacial acetic acid into 499.5 mL of water. Always add acid to water as it is an exothermic reaction.

Preparation of Gelatin-Coated Slides (if using):

The slides are prepared by placing clean slides in a slide rack and placing in a solution of ethanol for 2 minutes, then placing in distilled water for 2 minutes. The slides are then transferred to a 1% pig-skin gelatin solution, (Sigma: 300 Bloom) which has been heated to 65°C. Drain excess gelatin on paper towel and transfer to paraffin-free convection oven overnight at 60° C. After overnight drying the slides are ready to be used to mount fresh cut or paraffin sections.

Note: Commercially prepared slides can also be used. Our gelatin-coated slides provide an easy protocol for those that want to make their own slides that have maximum adhesion capabilities if properly prepared and dried. Fluoro-Jade has successfully been used with SuperFrost Gold slides as well as other makes of charged slides. For best results it is essential that cut sections be thoroughly dried on to any slide for best adhesion. Most failures stem from inadequate drying of the sections to slides.

Experimental Methods

1. FJC Staining Protocol: Non-Paraffin Embedded/4% PFA Frozen Sections

I. Mounting: Mount brain tissue sections on gelatin-coated or treated slides and dry at 50-60°C on a slide warmer for at least 30 minutes to 1 hour or even overnight for good adhesion and drying. It is critical that the tissue adhere thoroughly to the slide for best results. If using already mounted and dried slides start at step II.

II. Basic ethanol treatment (rehydration and permeabilization): Place 50 mL of Basic alcohol solution in a Coplin jar or other slide holding device. Place slides in Coplin jar and incubate slides for 5 minutes. Replace after every 20 slides.

III. Transfer the slides to a new Coplin jar containing a solution of freshly prepared 70% ethanol (in water) for 2 minutes. Replace after every 20 slides.

IV. Transfer slides to a new Coplin jar containing distilled water for 2 minutes. Replace water after every 20 slides.

V. Fluorescent background blocking & contrast optimization: Transfer slides to new Coplin jar or other device containing 0.06% potassium permanganate solution Incubate slides for up to 10 minutes. To halt staining, rinse slides for 2 minutes in fresh distilled water, change water every 20 slides. Potassium permanganate is a powerful fluorescent quencher. Timings in the potassium permanganate can be adjusted to optimize fluorescent background and signals. Some researchers use 0.04% permanganate for easier manipulation of fluorescent background.

VI. FJC Staining: Prepare sufficient quantity of FJC working solution for experiment (see above). Incubate slides in FJC working solution in a Coplin jar or suitable device in the dark or low light for 10 minutes.
OPTIONAL: DAPI nuclear counter stain: Use DAPI at 0.5-5 µg/mL, can be added to 1X FJC staining solution during dilution from 0.01% stock solution. Keep FJC jar covered with foil to protect it from light. Diluted dye is not stable for more than 4 hours at 2-8°C

VII. The slides are then rinsed for 3 x 1 minute with distilled water. Change water every 5-10 slides. Slides can be viewed immediately or dried; best photographs are typically with dried, permanently mounted slides.

VIII. The slides are dried on a slide warmer at 50-60°C for at least 5-10 minutes in the dark.

IX. The dry slides are then cleared by brief (1-5 minutes) immersion in xylene, in the dark.

X. Cover-slipping: The slides are then cover slipped with a non-aqueous, low fluorescent, styrene based mounting media, such as DPX. Mounting medias containing water or glycerol are not compatible, because their higher pH will cause FJC to come off targets.

XI. Visualization: Fluoro-Jade C labeled degenerating neurons are visualized with blue light excitation (like those used for visualizing fluorescein or FITC, see Fluorescent Imaging Settings), while DAPI counter stained cell nuclei are visualized with ultra-violet (UV) illumination. The resulting slides are quite stable and require no special storage conditions or anti-quench agents. Furthermore, the potassium permanganate pretreatment further enhances the permanence of the preparation resulting in extremely slow fading, even under high magnification.

2 FJC Staining Protocol: Paraffin Embedded Sections

The FJC staining protocol with paraffin sections is nearly identical to the protocol using non- treated sections (*Experimental Methods, Section 1*), the only differences are in the initial steps in which the paraffin is removed in two changes of xylene, followed by two changes of alcohol and subsequent rehydration through gradated alcohols. The basic ethanol treatment (*Experimental Methods, Section 1, Step II*) is not needed, as the xylene will also remove lipids.

I. Slides containing paraffin embedded sections of brain tissue are de-waxed in xylene 2X, then immersed in 100% ethanol for 2 x 5 min, then 70% alcohol for 2 minutes, and then rinsed with 2 x 1 minute changes of distilled water.

II. Transfer slides to a new Coplin jar containing distilled water for 2 minutes. This ensures that the slides are thoroughly hydrated. Replace water after every 20 slides.

III. Fluorescent background blocking & contrast optimization: Transfer slides to new Coplin jar or other device containing 0.06% potassium permanganate solution Incubate slides for up to 10 minutes. To halt staining, rinse slides for 2 minutes in fresh distilled water, change water every 20 slides. Potassium permanganate is a powerful fluorescent quencher. Timings in the potassium permanganate can be adjusted to optimize fluorescent background and signals. Some researchers use 0.04% permanganate for easier manipulation of fluorescent background.

IV. FJC Staining: Prepare sufficient quantity of FJC working solution for experiment (see above). Incubate slides in FJC working solution in a Coplin jar or suitable device in the dark or low light for 10 minutes.

V. OPTIONAL: DAPI nuclear counter stain: Use DAPI at 0.5-5 µg/mL, can be added to 1X FJC staining solution during dilution from 0.01% stock solution. Keep FJC jar covered with foil to protect it from light. Diluted dye is not stable for more than 4 hours at 2-8°C

VI. The slides are then rinsed for 3 x 1 minute with distilled water. Change water every 5-10 slides. Slides can be viewed immediately or dried; best photographs are typically with dried, permanently mounted slides.

VII. After the final rinse, excess water is removed by briefly draining the slides vertically on a paper towel (~15 sec).

VIII. The slides are then placed on a slide warmer, set at approximately 50 degrees C, until they are fully dry, (about 5-10 min) in the dark.

IX. The dry slides are then cleared by brief (1-5 minutes) by immersion in xylene, in the dark.

X. Cover-slipping: The slides are then cover slipped with a non-aqueous, low fluorescent, styrene based mounting media, such as DPX, a non-aqueous non-fluorescent plastic mounting media. Mounting medias containing water or glycerol are not compatible for long-term use, because their higher pH will cause FJC to come off targets.

XI. Visualization: Fluoro-Jade C labeled degenerating neurons are visualized with blue light excitation (like those used for visualizing fluorescein or FITC, see Fluorescent Imaging Settings), while DAPI counter stained cell nuclei are visualized with ultra-violet (UV) illumination. The resulting slides are quite stable and require no special storage conditions or anti-quench agents. Furthermore, the potassium permanganate pre-treatment further enhances the permanence of the preparation resulting in extremely slow fading, even under high magnification.

Technical Tips for Using Fluoro-Jade® C in Double-Labeling Experiments

Fluoro-Jade® C stain is compatible with a variety of immunohistochemical procedures including double-labeling with antibodies, detection of apoptosis with TUNEL, and many more. However, some modification and optimization of the basic protocols for these procedures is typically required by the investigator when combining FJC. Moreover, it is best to optimize the additional method first, before launching in to FJC staining to make sure that the conditions of the other method and the target detection have been optimized.

In general, we recommend with any immunohistochemical procedure being used in conjunction with FJC, that FJC staining be performed last for best results. Also, we recommend that labeling be performed on free-floating sections first, then sections transferred to gelatin or charged slides and dried down before beginning FJC staining. This will tend to preserve the structures better and “fix” the immune reagents on to the tissue in our experience, however, some of our customers have been successful with slide-mounted sections entirely, so one should likely optimize for what works best in the particularly laboratory and skill level.

Double-labeling with antibodies:

Because FJC is detected in the green channel, any suitable fluorescent dye that pairs well with FJC emission spectra can be used. For example, a red fluorescent Rhodamine (TRITC)-conjugated secondary antibody provides a good contrast with FJC dyes and is readily available *(see Appendix A for a simple antibody-FJC double label protocol)*

TUNEL-label considerations:

- For TUNEL-labeled sections, we strongly recommend omitting the potassium permanganate pretreatment, as it can cause background fluorescence in our experience.
- Because of the TUNEL-specific pre-treatments, the FJC tissue staining time will need to be optimized for each section set. Staining time is typically much longer, maybe 30 minutes to an hour with TUNEL treated materials, but trials will have to be done to ensure that the background is not too high.
- It is recommend rinsing the sections 3-4x in water, rather than just two times after long FJC staining incubations to help reduce background.

Additional Fluoro-Jade® C Technical Notes

1) Concerning the basic ethanol pretreatment (*Experimental Methods, Section 1, Step II*), this step can be omitted when using paraffin-processed tissue, as the ethanol treatment is part of the paraffin removal procedure. Slides must be fully rehydrated prior to the subsequent staining steps.

2) Concerning the potassium permanganate pretreatment, it is necessary because it allows for a significant reduction in background staining. However, it can also denature some antigenic epitopes and therefore, the time in this solution may need to be reduced when combining with immunofluorescent labeled tissue and sensitive epitopes.

3) Solvent-based slide drying: Traditionally, the sections are air dried on a slide warmer, since ethanol dehydration can produce a more diffuse dye stain. However, it is possible to solvent dehydrate the sections using butanol as follows: transfer sections to distilled water for 1 minute, then into a mixture of equal parts ethanol and butanol for 1 minute and then through 2 five-minute changes of butanol. The slides are then transferred to xylene and cover slipped as described above. Solvent dehydration allows for simultaneous processing of larger volumes of slides.

4) Alternative mounting media recipe. FJC and indeed FJ dyes in general bind their targets best under acidic conditions. A simple, acidic, non-permanent, **acidic coverslip mounting media** is: Mix 0.1% acetic acid and 80% glycerin in distilled water, check pH (pH = 4.5) and use. (taken from Ehara & Ueda (*Acta Histochem Cytochem.* 2009 Dec 29;42(6):171-9).

Positive Controls for FJC/FJB Studies

The exact entity that FJC binds to in degenerating neurons has not been resolved, and indeed even the exact conditions for its induction have not yet been truly understood. However, there is a vast amount of literature demonstrating the successful use of FJC in detecting degenerating neurons, so the phenomenon is real. It is important to note, however, that not all cases of neurodegeneration bring about positive FJC staining. Moreover, some common laboratory mouse strains are highly resistant to known positive control treatments such as kainic acid, and therefore can, in some instances, show no staining (see McKhann GM *et al.*, *Neuroscience.* 2003;122(2):551-61).

Furthermore, while neurodegeneration induced by proper treatment (e.g. kainic acid 10 mg/kg i.e.) can be used as positive controls, even with Kainic acid and susceptible animals there are pitfalls as Kainic acid (KA)-treated animals only work well if the correct timings are used. KA positive control animals need to exhibit seizure activity for at least 1 full hour and must survive for at least 1 day before sacrifice and must not be left to recover too long or FJC staining can be lost.

Thus typically, failure to observe FJC staining is not truly the fault of the dye or the method, but of the experimental system itself in many cases. As such often, positive controls can be difficult to generate, and depending upon the species, sometimes nearly impossible.

A possible solution for positive controls slides for FJC studies may adapted from this paper by Qiang G *et al*. (*Toxicol In Vitro.* 2014 Jun;28(4):469-72), where the authors found that various cell lines in culture when stressed with various drugs all stained strongly FJC positive, while untreated cells did not. Prepared coverslips or even paraffin blocks of such cell suspensions could potentially be used as positive control in research where they are needed.

Troubleshooting

FJC staining normally is very robust and almost faultless if conditions are suitable and the protocols have been followed. There are several excellent publications that review optimization methods for Fluoro-Jade® staining producers and at Biosensis we strongly recommend reviewing them. For instance, the publication by Ehara & Ueda (*Acta Histochem Cytochem.* 2009 Dec 29;42(6):171-9) is an excellent paper that not only covers animal pretreatments but troubleshooting of FJC staining as well. Some common difficulties include:

Problem: The tissue wrinkles or falls off slides when processing.
Solution: Use proper slide gelling procedure (see processing procedure described above); make sure tissue and slides are thoroughly dry and baked for best adhesion.

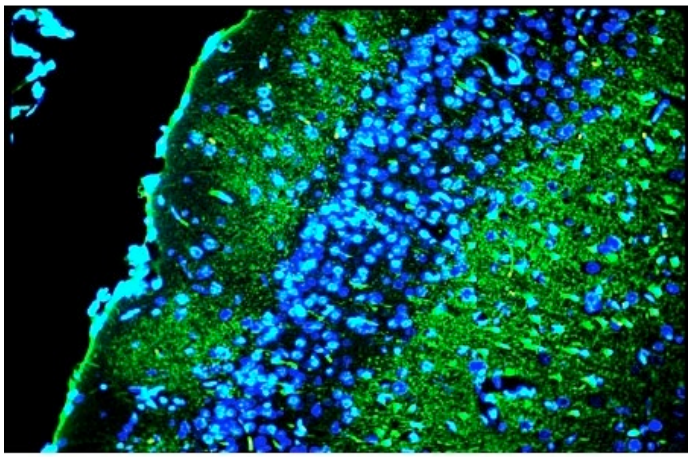
Problem: The staining is present but has low contrast (high background).
Solution: Reduce dye concentration or increase time in potassium permanganate.

Problem: The staining is present, but faint.
Solution: Increase the FJC concentration, or reduce time in potassium permanganate.

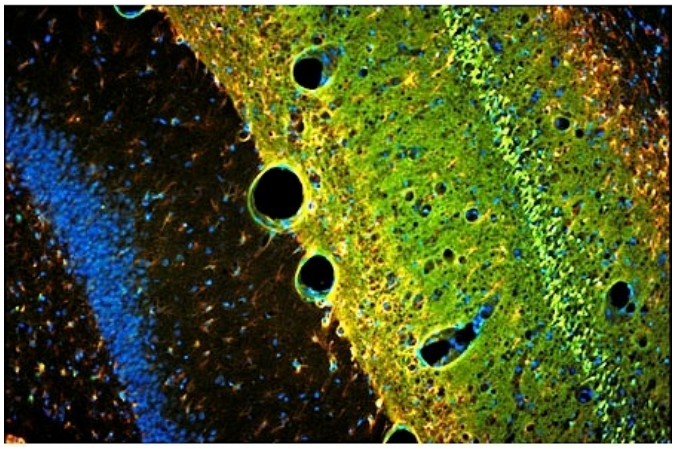
Problem: The stain is present after final rinse but lost following cover slipping.
Solution: Air dry slides rather than ethanol dehydration and avoid mounting media that contain polar solvents (e.g. water, ethanol, glycerin which interfere with FJC binding).

Problem: No staining observed.
Solution: Review the protocol steps; adjust the potassium permanganate timings and review the experimental design.

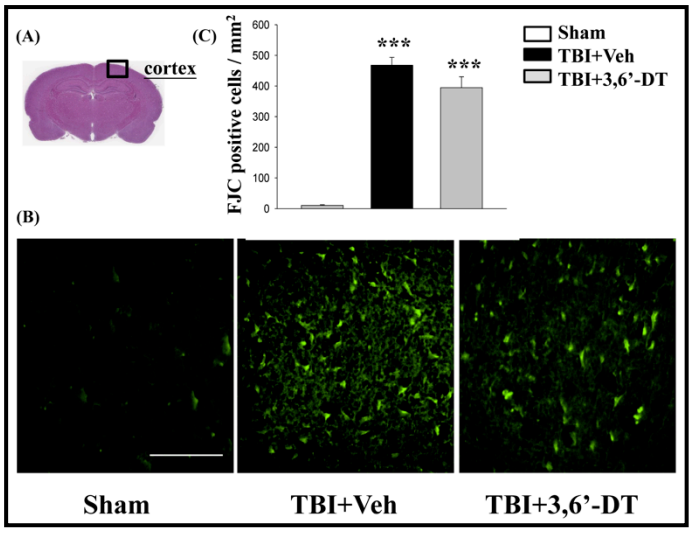
Example Images



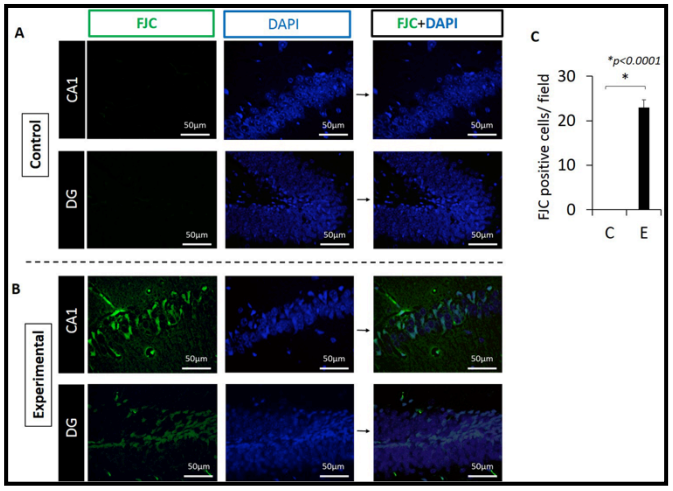
Double exposure using combined blue and ultraviolet epi-fluorescent illumination of the superficial layers of the cingulated rat cortex exposed to kainic acid. Layer I contain conspicuous Fluoro-Jade® C positive degenerating axon terminals. Layer II contains densely packed DAPI-positive viable granule cells. Layer III contains a mixture of Fluoro-Jade® C positive degenerating pyramidal cells and DAPI-positive viable pyramidal cells. Photo is courtesy of Dr. Larry Schmued.



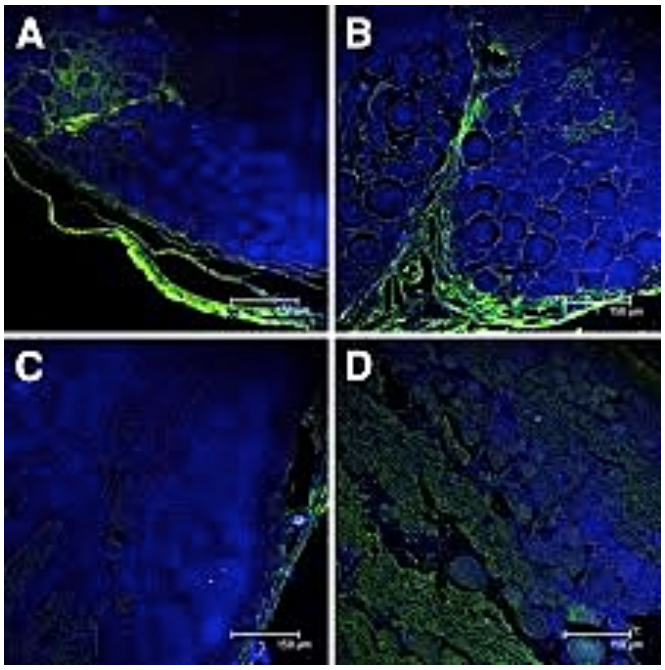
Triple exposure combining ultraviolet, blue and green light epi-fluorescent illumination (10X) of rat hippocampus exposed to kainic acid. The section was triple labeled with Fluoro-Jade® C and DAPI staining combined with GFAP immunohistochemistry. The section reveals extensive green Fluoro-Jade® C positive neuronal degeneration throughout the entire CA-1 region of the hippocampus. The underlying blue viable positive granule cells of the dentate gyrus are only DAPI positive. Both regions exhibit red GFAP positive hypertrophied astrocytes. Photo is courtesy of Dr. Larry Schmued.



TBI induces neuron degeneration within the contusion regions, and treatment with 3,6′-DT reduced the number of TBI-induced degenerating neurons. (A) Representative HE-stained coronal brain section from Sham that shows the area of evaluation. (B) Representative photomicrographs showing the presence of Fluoro-Jade® C (FJC)-staining at 24 h in different groups. (C) Quantitative comparison of mean densities of FJC-positive cells in the cortical contusion area at 24 h post-injury. Data are presented as mean ± S.E.M. (n = 5 in each group). *** $p < 0.001$ compared with the Sham group. Scale bar = 100 µm. Figure taken from Batsaikhan B *et al*., *Int J Mol Sci.* 2019 Jan 24;20(3).



Neurodegeneration is evident in the hippocampus in the experimental group. (A) CA1 and DG regions from control mouse. There is no FJC staining in these regions. (B) CA1 and DG regions which show FJC+ staining in representative section from an experimental mouse. Green: FJC staining indicating degenerating neurons. Blue: DAPI. (C) FJC+ cells are present in the experimental but not in control mice. Positive cells were counted in 220 µm x 175 µm rectangular areas. Representative of N = 4 mice/group. Figure taken from *PLoS One*. 2018 Oct 3;13(10): e0204941.



Neurodegeneration in inflamed DRG. Confocal microscope image of degenerating neurons (green) as stained by FJC in the DRG (Bb alone at 14 weeks) showing degenerating neuronal axons in a dorsal root, adjacent to the DRG are also seen to stain green with FJC. Nuclei of all cells appear blue due to staining with nuclear stain TOPRO3 (A), neurodegeneration in the DRG and dorsal root entering the DRG with BbþMel at 14 weeks (B), absence of neurodegeneration in an uninfected control rhesus macaque at 8 weeks (C), and presence of degenerating neurons in the DRG and areas corresponding to the dorsal roots that traverse the DRG in a section parallel to that showing neurodegeneration as seen with Bb alone at 14 weeks in Figure 4C (D). Bb, Borrelia burgdorferi; DRG, dorsal root ganglia; FJC, Fluoro-Jade® C; Mel, meloxicam. Figure taken from Ramesh G *et al*., *Am J Pathol*. 2015 May;185(5):1344-60.

Related Biosensis Products

Cat#	Product
TR-100-BG	Black-Gold® II Myelin RTD™ Staining Kit with Toluidine Blue O Counter Stain for Identifying Normal & Pathogenic Myelin
TR-100-FJ	Fluoro-Jade C (FJC) Ready-to-Dilute Staining Kit for identifying Degenerating Neurons
TR-100-FJT	Fluoro-Jade C (FJC) Ready-to-Dilute Staining Kit for identifying Degenerating Neurons (Trial size)
TR-150-FJB	Fluoro-Jade® B (FJB) Powder for identifying Degenerating Neurons
TR-300-AG	Amylo-Glo® RTD™ Amyloid Plaque Stain Reagent
TR-400-AG	Amylo-Glo® RTD™ Amyloid Plaque Stain Reagent with EtBr Counter Stain
TR-700-HQO	HQ-O RTD™ Amyloid Plaque Stain Reagent
TR-700-HQOT	HQ-O RTD™ Amyloid Plaque Stain Reagent (Trial size)

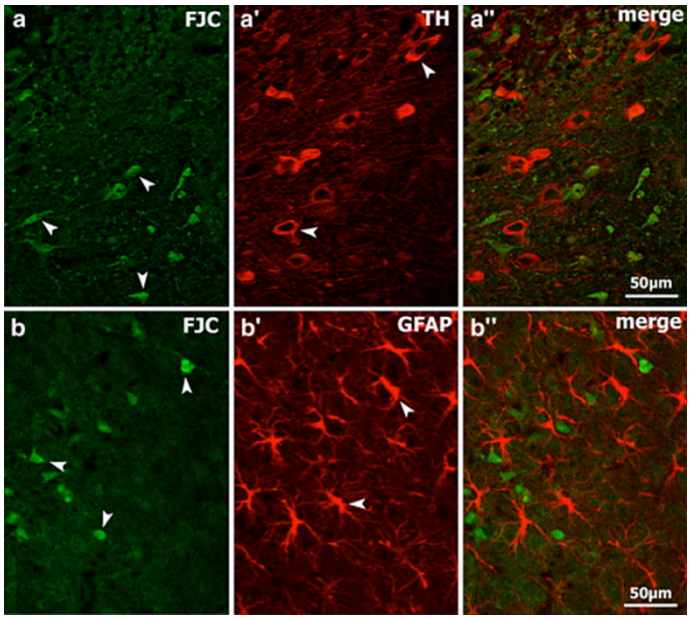
Appendix A, Sample Protocol: Double-Labeling with FJC and GFAP Antibody

I. Prepared free-floating tissue sections are incubated in a prediluted solution of anti-GFAP antibody (e.g., Biosensis cat# M-1375-100, C-1373-50) at 2-8°C, covered, in a humid chamber in the refrigerator for 1-3 days. Note GFAP is an internal cellular marker, so permeabilization or antigen recovery will be necessary for some systems.

II. Sections are rinsed in two changes of PBS for 10 min each and then transferred to a tetramethylrhodamine isothiocynate (TRITC) labeled secondary antibody for 1 h at room temperature.

III. Sections are rinsed in two changes of PBS for 10 min each and then the sections are mounted onto gelled slides from distilled water and air dried, covered on a slide warmer at 50°C for 30 min, until thoroughly dry.

IV. To combine with Fluoro-Jade® C, the slide mounted sections are then rehydrated for 2 min in distilled water and the FJC staining procedure is followed as described in *Sections 1 or 2* above starting at step the potassium permanganate blocking step. It is worth mentioning that the incubation time in potassium permanganate may need to be reduced when co-localizing those antigenic epitopes susceptible to chemical oxidation.



Neurodegeneration in the substantia nigra visualized with FJC (green). Double-labelling with either antibody to TH (top panel, red) or GFAP (lower panel, red) demonstrates compatibility of FJC in antibody multiple-label fluorescent studies. Figure taken from Bian GL *et al.*, *Brain Res.* 2007 May 30; 1150:55-61.

Appendix B: Comparison between Aqueous and Non-Aqueous Mounting Media

Positive control tissue sections were stained with Fluoro-Jade® C and then air-dried on a slide warmer. Then, one slide was cleared in xylene and coverslipped with DPX (non-aqueous mounting media), while the other was coverslipped with glycerin and distilled water (2:1), an aqueous mounting media.

The images below compare FJC-labeled degenerating perirhinal cortical neurons. Although degenerating neurons can be easily localized following coverslipping with either mounting media, there is a definite qualitative difference between the two. Specifically, coverslipping with DPX results in the neurons appearing smaller and exhibiting relatively high contrast. The stained neurons coverslipped with aqueous mounting media (glycerin and distilled water) generally appear larger and exhibit lower contrast. Both stains label proximal dendrites in addition to their cell bodies.