SUPPLEMENTAL PROTOCOL

# PHA-L Method For Tracing Efferent Neuronal Projections

This procedure was kindly supplied by Dr. Charles R. Gerfen, Laboratory of Cell Biology, NIMH, Bethesda, MD 20892

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## Reference

Gerfen CR, et al. 2016. An Anterograde Neuroanatomical Tracing Method That Shows the Detailed Morphology of Neurons, Their Axons and Terminals: Immunohistochemical Localization of an Axonally Transported Plant Lectin, Phaseolus Vulgaris-Leucoagglutinin (PHA-L). *Brain Research.*

## A. Solutions:

1. PHA-L (Cat.No. L-1110): 2.5% solution (5 mg/200 µl) in sodium phosphate buffered saline (NaPBS). Dissolve by adding 10 mM phosphate, pH 8 (0.14 g $Na_2HP0_4$ in 100 ml distilled water, pH adjusted with diluted HCl), to the lectin. Gently rotate until the solution is clear. The PHA-L solution can be divided into 50 µl aliquots and stored frozen. Repeated freeze-thawing may inactivate the lectin.
2. KPBS: 0.02 M potassium phosphate buffered saline, pH 7.4
   - Prepare solution A: 0.5 M potassium phosphate dibasic ($K_2HP0_4$) 87 g/1000 ml
   - Prepare solution B: 0.5 M potassium phosphate monobasic ($KH_2PO_4$) 68 g/1000 ml
   - Prepare KPBS, mix: 32.8 ml solution A + 7.2 ml solution B + 960 ml $H_20$ + 9 g NaCl

## B. Iontophoretic Injection:

To obtain selective neuronal labeling, the PHA-L should be delivered iontophoretically.

Injections are made through a micropipette with a 10–15 µm tip diameter. 5 µAmp positive current, pulsed (on for 7 sec, off for 7 sec), for 15–20 min is used to inject the tracer. A Midgard Model CS-3 constant current device can be used.

Micropipettes with a fine glass thread to facilitate filling are used (O.D.: l mm; I.D.: 0.58 mm). The PHA-L solution is loaded by drawing the lectin solution into the tip via a vacuum, and then the pipettes are backfilled sufficiently to allow contact with the electrode wire. A silver wire, inserted into the PHA-L solution in the micropipette, is connected to the positive terminal of a constant current device. The negative terminal is used to ground the animal via a connection to either the animal's tail or some other part of the body.

**Note:** The injection parameters outlined above must be adhered to quite rigidly. Pipette tip diameters greater than 15 µm, injection currents greater than 5 µA, and (especially) conventional pressure injections of the lectin all diminish the resolution afforded by the method regarding the filling of cells and axons, and retrograde transport may be enhanced by these procedures.



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## C. Survival Period:

An estimate for PHA-L anterograde transport is about 4–6 mm/day. Survival periods of over 18 days have been used with no apparent degradation of the tracer. It is advisable to use a survival period that is sufficient to ensure labeling of the longest pathway to be investigated.

The lectin appears to be taken up and fills neurons at the injection site during or immediately after injection. The population of filled neurons does not appear to change between 15 min and 14 days. There is usually a zone of diffusion of the tracer around the filled neurons. This zone appears to increase with survival time but does not appear to contribute to the labeling of filled axons or their terminals. The zone of diffusion should not be confused with the effective injection site.

## D. Perfusion:

Various perfusion methods may be used. The following is the one found to provide optimal labeling.

Sequential perfusion with animal on ice with:

a. Normal saline (100–150 ml)

b. A suitable paraformaldehyde fixative, such as $IO_4$/lysine/paraformaldehyde, 150 ml, 10 min, 4°C. (McLean IW, et al. 1974. Periodate-Lysine-Paraformaldehyde Fixative A New Fixative for Immunoelectron Microscopy. *Journal of Histochemistry & Cytochemistry*.)

c. Post-fix brain in perfusion fixative overnight at 4°C.

## E. Staining Sections [using Anti-PHA (E+L), made in goat, Cat. No. AS-2224]:

1. Frozen or Vibratome sections, 30 µm thick, are collected in ice cold KPBS.
2. Sections are incubated overnight in KPBS/0.3% Triton X-100 + 2% normal rabbit serum (NRS, Cat. No. S-5000). If Triton X-100 is used in this step, the detergent can be deleted from diluents in subsequent steps. Triton X-100 concentrations up to 1.0% can be used.
3. Incubate sections in 2 µg/ml (1:1000 dilution of reconstituted antibody solution) affinity-purified goat anti-PHA (E+L) (Cat. No. AS-2224) in KPBS/2% NRS for 48 h at 4°C with constant, gentle agitation. Sodium azide (0.1%) can also be added as a preservative.



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## F. Staining Sections [using Anti-PHA (E+L), made in rabbit, Cat. No. AS-2300]:

1. Frozen or Vibratome sections, 30 µm thick, are collected in ice cold KPBS.
2. Sections are incubated overnight in KPBS/0.3% Triton X-100 + 2% normal goat serum (NGS, Cat. No. S-1000). If Triton X-100 is used in this step, the detergent can be deleted from diluents in subsequent steps. Triton X-100 concentrations up to 1.0% can be used.
3. Incubate sections in 2 µg/ml (1:1000 dilution of reconstituted antibody solution) affinity-purified rabbit Anti-PHA (E+L) (Cat. No. AS-2300) in KPBS/2% NGS for 48 h at 4°C with constant, gentle agitation. Sodium azide (0.1%) can also be added as a preservative.

   After incubation in primary antibody to PHA-L, sections are then processed for either immunofluorescence or immunoperoxidase labeling.

## Immunoperoxidase Procedure Using the VECTASTAIN® ABC Kit*

Cat. No. PK-4001 [for rabbit Anti-PHA (E+L)] or PK-4005 [for goat Anti-PHA(E+L)]

4. Rinse 2 x l0 min with KPBS/2% appropriate normal serum.
5. Incubate sections for 45–60 min in diluted biotinylated affinity-purified goat Anti-Rabbit IgG (H+L) or rabbit Anti-Goat IgG (H+L) (one drop biotinylated antibody per 10 ml of KPBS/2% appropriate normal serum/0.3% Triton X-l00).
6. Rinse 2 x l0 min with KPBS/2% appropriate normal serum.
7. Incubate sections for 45 min in the VECTASTAIN ABC solution prepared as in kit instructions using KPBS (no serum should be added).
8. Rinse 2 x l0 min with KPBS/2% appropriate normal serum.
9. Repeat step 5 for 30 min using same solution.
10. Rinse 2 x l0 min with KPBS.
11. Repeat step 7 for 30 min using same solution.
12. Rinse 3 x l0 min with KPBS.
13. Incubate sections for 5–45 min in freshly mixed, filtered solution containing: 0.05% diaminobenzidine tetrahydrochloride + 0.004% hydrogen peroxide in KPBS or use the DAB Substrate Kit (Cat.No. SK-4100) or ImmPACT® DAB (Cat.No. SK-4105).
14. Rinse 5 x 5 min with KPBS.



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15. Mount sections onto chromium aluminum coated or VECTABOND® Reagent-treated slides from KPBS. Air dry.

16. Sections may then be left uncounterstained or be counterstained using thionin. In either case, sections are defatted in 2 x 30 min rinses of 1:1 chloroform:alcohol. Those to be left uncounterstained are rinsed in 2 x 5 min changes of 100% alcohol, followed by immersion in 2 x 5 min changes of xylene and coverslipped. Those to be counterstained are hydrated through descending concentrations of alcohol, stained with thionin or neutral red, and then dehydrated and coverslipped out of xylene.

*The VECTASTAIN® ABC Kit (Goat IgG), Cat. No. PK-4005, consists of 2 ml each of the A Reagent (Avidin DH) and B Reagent (Biotinylated Horseradish Peroxidase). Also included are 1 ml of affinity-purified Biotinylated Rabbit Anti-Goat IgG (H+L) and 3 ml of normal rabbit serum. The VECTASTAIN® ABC Kit (Rabbit IgG) Cat. No. PK-4001, consists of the same A Reagent and B Reagent as above but contains 1 ml of affinity-purified Biotinylated Goat Anti-Rabbit IgG (H+L) and 3 ml of normal goat serum.

**Note:** If VECTASTAIN® Elite® ABC kits (Cat. No. PK-6101 or PK-6105) are used for detection, a significant increase in sensitivity can be achieved.

## Fluorescent Staining

Biotinylated Anti-Goat IgG or Biotinylated Anti-Rabbit IgG supplied in the VECTASTAIN ABC Kits can be used as described in step 5 or the biotinylated antibody can be purchased separately as Cat. No. BA-5000 (for goat primary antibody) or BA-1000 (for rabbit primary antibody), and used at 10 µg/ml. For fluorescent staining, substitute the following for steps 7 thru 9:

7a. Incubate sections in freshly diluted 20 µg/ml fluorescein Avidin DCS (Cat. No. A-2011) in 0.02M KPBS/0.3% Triton X-l00 for 45 min.

8a. Rinse 2 x l0 min in KPBS.

9a. Mount with VECTASHIELD mounting medium and coverslip.

**Note:** Because different information can be derived from sections stained with peroxidase and fluorescence, it may be advisable to process alternate sections through the injection site for each procedure.

In cutting a brain, two adjacent series of sections through the brain are saved, except at the injection site where additional sections are saved. These few additional sections can be processed for immunofluorescence staining using a shortened staining protocol that involves 45–60 min incubation in a 2 µg/ml solution of anti-PHA-L, a wash, followed by 30–45 min in a 10 µg/ml solution of biotinylated secondary antibody, a wash, and 30 min in 20 µg/ml fluorescein Avidin DCS. This allows a preliminary determination of the injection placement to be made and facilitates the decision as to whether the time and energy should be expended in processing a complete series for immunofluorescence, immunoperoxidase, or both.

Additionally, when the PHA-L technique is used in combination with other methods, the labeling procedures obviously must be matched for the combination procedure. Thus, combined use of retrogradely-transported dyes necessitates use of immunofluorescent localization of PHA-L while combinations with autoradiography necessitate the use of the peroxidase protocol.





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