THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Approved Biohazards Subcommittee: September 25, 2009
Biosafety Website: www.uwo.ca/humanresources/biosafety/

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario or in charge of a laboratory/facility where the use of Level 1, 2 or 3 biohazardous agents is described in the laboratory or animal work proposed. The form must also be completed if any work is proposed involving animals carrying zoonotic agents infectious to humans or involving plants, fungi, or insects that require Public Health Agency of Canada (PHAC) or Canadian Food Inspection Agency (CFIA) permits.

This form must be updated at least every 3 years or when there are changes to the biohazards being used.

Containment Levels will be established in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Public Health Agency of Canada (PHAC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety, (OHS), (Support Services Building, Room 4190) for distribution to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Officer at extension 81135 or biosafety@uwo.ca. If there are changes to the information on this form (excluding grant title and funding agencies), contact Occupational Health and Safety for a modification form. See website: www.uwo.ca/humanresources/biosafety

PRINCIPAL INVESTIGATOR: JOHN CIRIELLO
SIGNATURE:
DEPARTMENT: PHYSIOLOGY / PHARMACOLOGY
ADDRESS: SCHULICH SCHOOL MED/DENT.
PHONE NUMBER: 519-661-3484
EMERGENCY PHONE NUMBER(S):
EMAIL: john.ciriello@schulich.uwo.ca

Location of experimental work to be carried out: Building(s) DENTAL SCI Room(s) ~~2004~~ 2005 DSB

*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to its being sent to the University of Western Ontario Biosafety Officer (See Section 12.0, Approvals).

FUNDING AGENCY/AGENCIES: HSFO
GRANT TITLE(S): CENTRAL MECHANISMS IN THE PATHOGENESIS OF HYPERTENSION

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED. A GRANT SUMMARY PAGE MAYBE ADEQUATE IF IT PROVIDES SUFFICIENT DETAIL ABOUT EACH BIOHAZARD USED. NO BIOHAZARD USED!

Names of all personnel working under Principal Investigators supervision in this location:

JASON MOREAU
WASEEM IQBAL
MEGAN MICHELS

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

1.0 Microorganisms

1.1 Does your work involve the use of biological agents? O YES ⊗ NO
(including but not limited to bacteria and other microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)? If no, please proceed to Section 2.0

Do you use microorganisms that require a permit from the CFIA? O YES ⊗ NO
If YES, please give the name of the species. _____
What is the origin of the microorganism(s)? _____
Please describe the risk (if any) of escape and how this will be mitigated:

_____
_____
_____

Please attach the CFIA permit.
Please describe any CFIA permit conditions:

_____
_____
_____

1.2 Please complete the table below: NA

Name of Biological agent(s)*	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent? YES/NO	Maximum quantity to be cultured at one time? (in Litres)	Source/ Supplier	PHAC or CFIA Containment Level
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3
	O Yes O No	O Yes O No	O Yes O No			O 1 O 2 O 3

*Please attach a Material Safety Data Sheet or equivalent from the supplier.

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? O YES ⊗ NO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (i.e. derived from fresh tissue) that will be grown in culture: NA

Cell Type	Is this cell type used in your work?	Source of Primary Cell Culture Tissue	AUS Protocol Number
Human	O Yes O No		Not applicable
Rodent	O Yes O No		
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

2.3 Please indicate the type of established cells that will be grown in culture in: NA

Cell Type	Is this cell type used in your work?	Specific cell line(s)*	Supplier / Source
Human	O Yes O No		
Rodent	O Yes O No		
Non-human primate	O Yes O No		
Other (specify)	O Yes O No		

*Please attach a Material Safety Data Sheet or equivalent from the supplier. (For more information, see www.atcc.org)

2.4 For above named cell types(s) indicate PHAC or CFIA containment level required O 1 O 2 O 3

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? O YES O NO
If no, please proceed to Section 4.0

3.2 Indicate in the table below the Human Source Material to be used. NA

Human Source Material	**Source/Supplier /Company Name**	**Is Human Source Material Infected With An Infectious Agent? YES/NO**	**Name of Infectious Agent (If applicable)**	**PHAC or CFIA Containment Level (Select one)**
Human Blood (whole) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Blood (fraction) or other Body Fluid		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (unpreserved)		O Yes O No O Unknown		O 1 O 2 O 3
Human Organs or Tissues (preserved)		Not Applicable		Not Applicable

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents, or cells described in Sections 1.0 and 2.0? O YES O NO If no, please proceed to Section 5.0

4.2 Will genetic modification(s) involving plasmids be done? O YES, complete table below O NO

Bacteria Used for Cloning *	Plasmid(s) *	Source of Plasmid	Gene Transfected	Describe the change that results

* Please attach a Material Data Sheet or equivalent if available.

4.3 Will genetic modification(s) involving viral vectors be made? O YES, complete table below O NO

Virus Used for Vector Construction	Vector(s) *	Source of Vector	Gene(s) Transduced	Describe the change that results

* Please attach a Material Safety Data Sheet or equivalent.

4.4 Will genetic sequences from the following be involved?

- HIV O YES, please specify _____ O NO
- HTLV 1 or 2 or genes from any Level 1 or Level 2 pathogens O YES, specify _____ O NO
- SV 40 Large T antigen O YES O NO
- E1A oncogene O YES O NO
- Known oncogenes O YES, please specify _____ O NO
- Other human or animal pathogen and or their toxins O YES, please specify _____ O NO

4.5 Will virus be replication defective? O YES O NO

4.6 Will virus be infectious to humans or animals? O YES O NO

4.7 Will this be expected to increase the containment level required? O YES O NO

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials be conducted involving a biological agent? O YES X NO
(including but not limited to microorganisms, viruses, prions, parasites or pathogens of plant or animal origin)
If no, please proceed to Section 6.0

5.2 If YES, please specify which biological agent will be used: _____
Please attach a full description of the biological agent.

5.2 Will the biological agent be able to replicate in the host? O YES O NO

5.3 How will the biological agent be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? O YES, number: _____ O NO O PENDING

6.0 Animal Experiments

6.1 Will live animals be used? X YES O NO If no, please proceed to section 7.0

6.2 Name of animal species to be used RAT

6.3 AUS protocol # 2008-030

6.4 Will any of the agents listed in section 4.0 be used in live animals O YES, specify: _____ O NO

6.5 Will the agent(s) be shed by the animal: O YES X NO, please justify:

_____

_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

7.0 Use of Animal species with Zoonotic Hazards NA

7.1 Will any of the following animals or their organs, tissues, lavages or other body fluids including blood be used?

- Pound source dogs O YES O NO
- Pound source cats O YES O NO
- Cattle, sheep or goats O YES O NO
- Non-human primates O YES, please specify species _____ O NO
- Wild caught animals O YES, please specify species & colony # _____ O NO
- Birds O YES O NO
- Others (wild or domestic) O YES, please specify _____ O NO

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? ☒ YES O NO If no, please proceed to Section 9.0

8.2 If YES, please name the toxin(s) Cholera-Toxin subunit B (non-toxic) - see attached paper.
Please attach information, such as a Material Safety Data Sheet, for the toxin(s) used.

8.3 What is the LD_{50} (specify species) of the toxin non-toxic

8.4 How much of the toxin is handled at one time*? 2-10 ug

8.5 How much of the toxin is stored*? 100 ug

8.6 Will any biological toxins be used in live animals? O YES, Please provide detais:_____ ☒ NO

*For information on biosecurity requirements, please see:
http://www.uwo.ca/humanresources/docandform/docs/healthandsafety/biosafety/Biosecurity_Requirements.pdf

9.0 Insects Requiring CFIA Permits NA

9.1 Do you use insects that require a permit from the CFIA? O YES ☒ NO
If no, please proceed to Section 10.0

9.2 If YES, please give the name of the species. _____

9.3 What is the origin of the insect? _____

9.4 What is the life stage of the insect? _____

9.5 What is your intention? O Initiate and maintain colony, give location: _____
O "One-time" use, give location: _____

9.6 Please describe the risk (if any) of escape and how this will be mitigated:
_____
_____
_____

9.7 Please attach the CFIA permit.

9.8 Please describe any CFIA permit conditions:
_____
_____

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

10.0 Plants Requiring CFIA Permits NA

10.1 Do you use plants that require a permit from the CFIA? O YES O NO
If no, please proceed to Section 11.0

10.2 If YES, please give the name of the species. _____

10.3 What is the origin of the plant? _____

10.4 What is the form of the plant (seed, seedling, plant, tree...)? _____

10.5 What is your intention? O Grow and maintain a crop O "One-time" use

10.6 Do you do any modifications to the plant? O YES O NO
If yes, please describe: _____
_____

10.7 Please describe the risk (if any) of loss of the material from the lab and how this will be mitigated:
_____
_____
_____

10.8 Is the CFIA permit attached? O YES O NO
If NO, please forward the permit to he Biosafety Officer when available.

10.9 Please describe any CFIA permit conditions:
_____
_____
_____

11.0 Import Requirements NA

11.1 Will any of the above agents be imported? O YES, please give country of origin_____
If no, please proceed to Section 12.0 O NO

11.2 Has an Import Permit been obtained from HC for human pathogens? O YES O NO

11.3 Has an import permit been obtained from CFIA for animal or plant pathogens? O YES O NO

11.4 Has the import permit been sent to OHS? O YES, please provide permit # _____ O NO

12.0 Training Requirements for Personnel Named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS:

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS (Western or equivalent)
- Employee Health and Safety Orientation

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____ Feb. 18/10

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

13.0 Containment Levels NA

11.1 For the work described in sections 1.0 to 9.0, please indicate the highest HC or CFIA Containment Level required. O 1 O 2 O 3

13.2 Has the facility been certified by OHS for this level of containment?

O YES, permit # if on-campus_____
O NO, please certify
O NOT REQUIRED for Level 1 containment

14.0 Procedures to be Followed

14.1 As the Principal Investigator, I will ensure that this project will follow the Western Biosafety Guidelines and Procedures Manual for Containment Level 1 & 2 Laboratories (and the Level 3 Facilities Manual for Level 3 projects). I will ensure that UWO faculty, staff and students working in my laboratory have an up-to-date Hazard Communication Form, found at http://www.wph.uwo.ca/

SIGNATURE _____ Date: Feb. 18/10

14.2 Please describe additional risk reduction measures will be taken beyond containment level 1, 2, or 3 measures, that are unique to this agent.

_____
_____
_____

14.3 Please outline what will be done if there is an exposure to the biohazards listed, such as a needlestick injury:

_____
_____

15.0 Approvals

UWO Biohazard Subcommittee: SIGNATURE: _____
Date:_____

Safety Officer for Institution where experiments will take place: SIGNATURE:_____
Date:_____

Safety Officer for University of Western Ontario (if different from above): SIGNATURE:_____
Date:_____

Approval Number: _____ Expiry Date (3 years from Approval): _____

Special Conditions of Approval:

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

Hi Jennifer:
As shown in the paper I attached to the submission, we inject micro-iontophoretically cholera-toxin subunit B conjugated to horseradish peroxidase or a fluorsecent dye stereotaxically into specific brain areas in anesthetized rats. Animals are allowed to recover and after a survival time of 4-14 days (depending on length of central pathway being examined) the animal is sacrificed, brain fixed, removed, sectioned at 50 um and tissue processed histochemically or immunohistochemically for visualization of pathway in question.

Hope this helps.
John

Dr. John Ciriello
Professor
Department of Physiology and Pharmacology
Schulich School of Medicine and Dentistry
Health Sciences Centre
University of Western Ontario
London, ON, N6A 5C1
Canada
Fax: 519-661-3827
Tel: 519-661-3484
E-mail: john.ciriello@schulich.uwo.ca

Cholera Toxin Subunit B (CT-B) Conjugates

Quick Facts

Storage upon receipt:

- ≤ –20°C
- Desiccate
- Protect from light

Abs/Em: See Table 1

Table 1. Conjugates of cholera toxin subunit B.

Label	Abs/Em *	Unit Size	
		100 µg	500 µg
Alexa Fluor 488	495/519	C-34775	C-22841
Alexa Fluor 555	555/565	C-34776	C-22843
Alexa Fluor 594	590/617	C-34777	C-22842
Alexa Fluor 647	650/668	C-34778	
Biotin	NA	C-34779	
Horseradish peroxidase (HRP)	NA	C-34780	

* Approximate absorption (Abs) and fluorescence emission (Em) maxima, in nm. NA = Not applicable.

Introduction

Cholera toxin from *Vibrio cholerae* is comprised of two subunits, A and B, arranged in an AB_5 configuration. The A subunit is an ADP-ribosyltransferase, which disrupts the proper signaling of G proteins and eventually leads to dehydration of the cell.[1] The nontoxic B subunit is important to the protein complex as it allows the protein to bind to cellular surfaces via the pentasaccharide chain of ganglioside G_{M1}.[2] At neutral pH, the 11.4 kDa B subunit exists as a 57 kDa pentamer.[3]

The B subunit of cholera toxin (CT-B) has proven to be a powerful tool for retrograde labeling of neurons.[4,5] This tracer has been used in a variety of applications, including tracing of rat forebrain afferents,[6] projections of the parabrachial region[7] and neurons of the urinary bladder wall.[8] More recently, researchers have found that CT-B can be used as a marker for lipid rafts, which are membrane microdomains enriched in cholesterol and sphingolipids. Lipid rafts segregate specific groups of proteins and thereby provide a hub for cellular signaling and protein trafficking.[9, 10]

Molecular Probes' CT-B conjugates (Table 1) are made from recombinant cholera toxin subunit B. Because the B-subunit source material is recombinant in origin, it is extremely pure and completely free of the toxic A subunit.

Materials

The biotin- and dye-labeled cholera toxin B subunits are supplied in unit sizes of 100 µg and 500 µg. When stored desiccated at ≤–20°C, these products are stable for at least six months. Solutions of 1.0 mg/mL can be prepared by dissolving the powder in 0.1 mL or 0.5 mL of buffer, for example, phosphate-buffered saline (PBS). With the addition of 2 mM sodium azide, solutions can be stored at 2–6°C for approximately three months. For longer storage, divide the solution into aliquots and freeze at ≤–20°C.

The peroxidase conjugates of cholera toxin B are supplied in a unit size of 100 µg. When stored desiccated at –20°C, the lyophilized powder is stable for at least six months. Solutions of 1.0 mg/mL can be prepared by dissolving the powder in 0.1 mL of PBS, pH 7.2. Store solutions at 2–6°C with the addition of thimerosal to a final concentration of 0.02%. DO NOT USE AZIDE for the HRP-conjugates. For prolonged storage after reconstitution, add glycerol to a final concentration of 50% (v/v), aliquot and store at –20°C. When stored properly, solutions are stable for approximately three months. PROTECT FROM LIGHT. AVOID REPEATED FREEZING AND THAWING OF SOLUTIONS.

For the Alexa Fluor and biotin conjugates, the lot-specific degree of labeling (typically 5–10 moles of dye per mole of the B subunit pentamer) is indicated on the product label.

Application

Due to the diversity of applications for the cholera toxin subunit B, please consult the primary literature for appropriate working concentrations.

References

1. J Biol Chem 255, 1252 (1980); 2. Mol Microbiol 13, 745 (1994); 3. Biochemistry 35, 16069 (1996); 4. Brain Res 243 215 (1982); 5. Brain Res 231, 33 (1982); 6. Neuroscience 82, 443 (1998); 7. Brain Res 816, 364 (1999); 8. Neuroscience 87, 275 (1998); 9. J Cell Biol 147, 447 (1999); 10. J Cell Biol 141, 929 (1998).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
C-34775	cholera toxin subunit B (recombinant), Alexa Fluor® 488 conjugate	100 µg
C-22841	cholera toxin subunit B (recombinant), Alexa Fluor® 488 conjugate	500 µg
C-34776	cholera toxin subunit B (recombinant), Alexa Fluor® 555 conjugate	100 µg
C-22843	cholera toxin subunit B (recombinant), Alexa Fluor® 555 conjugate	500 µg
C-34777	cholera toxin subunit B (recombinant), Alexa Fluor® 594 conjugate	100 µg
C-22842	cholera toxin subunit B (recombinant), Alexa Fluor® 594 conjugate	500 µg
C-34778	cholera toxin subunit B (recombinant), Alexa Fluor® 647 conjugate	100 µg
C-34779	cholera toxin subunit B (recombinant), biotin-XX conjugate	100 µg
C-34780	cholera toxin subunit B (recombinant), horseradish peroxidase conjugate	100 µg

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site – **www.probes.com** – for the most up-to-date information

Molecular Probes, Inc.
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 344-6504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 465-8338 • Fax: (541) 344-6504 • order@probes.com

Toll-Free Ordering for USA and Canada:
Order Phone: (800) 438-2209 • Order Fax: (800) 438-0228

Technical Assistance: 8:00 am to 4:00 pm (Pacific Time)
Phone: (541) 465-8353 • Fax: (541) 465-4593 • tech@probes.com

Molecular Probes Europe BV
Poortgebouw, Rijnsburgerweg 10
2333 AA Leiden, The Netherlands
Phone: +31-71-5233378 • Fax: +31-71-5233419

Customer Service: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5236850 • Fax: +31-71-5233419
eurorder@probes.nl

Technical Assistance: 9:00 to 16:30 (Central European Time)
Phone: +31-71-5233431 • Fax: +31-71-5241883
eurotech@probes.nl

Molecular Probes' products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Several of Molecular Probes' products and product applications are covered by U.S. and foreign patents and patents pending. Our products are not available for resale or other commercial uses without a specific agreement from Molecular Probes, Inc. We welcome inquiries about licensing the use of our dyes, trademarks or technologies. Please submit inquiries by e-mail to busdev@probes.com. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Multiple neuroanatomical tract-tracing using fluorescent Alexa Fluor conjugates of cholera toxin subunit B in rats

William L Conte[1,2], Hiroaki Kamishina[3] & Roger L Reep[1,2]

[1]Department of Physiological Sciences, University of Florida, Gainesville, Florida, USA. [2]McKnight Brain Institute, University of Florida, Gainesville, Florida, USA. [3]Department of Veterinary Medicine, Iwate University, Morioka, Japan. Correspondence should be addressed to W.L.C. (billy.conte@mbi.ufl.edu).

Published online 16 July 2009; doi:10.1038/nprot.2009.93

Cholera toxin subunit B (CTB) is a highly sensitive retrograde neuroanatomical tracer. With the new availability of fluorescent Alexa Fluor (AF) conjugates of CTB, multiple neuroanatomical connections can be reliably studied and compared in the same animal. Here we provide a protocol that describes the use of AF-CTB for studying connections in the central nervous system of rats. The viscous properties of CTB allow small and discreet injection sites yet still show robust retrograde labeling. Furthermore, the AF conjugates are extremely bright and photostable, compared with other conventional fluorescent tracers. This protocol can also be adapted for use with other neuroanatomical tracers. Including a 7-d survival period, this protocol takes ~11 to 12 d to complete in its entirety.

INTRODUCTION

It is desirable to compare multiple connections within the same brain in order to define topographic relationships, to assess the extent of convergence or divergence and to assess changes in connections after experimental manipulations. One way of doing this is to use multiple neuroanatomical tracer types in the same brain. Often, the use of different tracers causes interpretation problems, because there is variability in the quality of uptake and transport, tendency to spread in the tissue and capacity to produce local necrosis by different tracers. Thus, it is extremely beneficial to use the same type of neuroanatomical tracers in order to avoid any potential confounding factors. Although this has long been possible with conventional fluorescent-labeled axonal tracers, the usefulness of fluorescent dyes has been limited due to the less-than-optimal signal intensity and quick photobleaching. Nonfluorescent-based double labeling relies on using antibodies to differentiate between two different tracers, and can often be time-intensive and costly.

Historically, the use of the neuroanatomical tracer cholera toxin subunit B (CTB) has been limited to single-labeling techniques using bright-field horseradish peroxidase and DAB staining[1–4]. Recently, CTB has been made available conjugated with the Alexa Fluor (AF) fluorescent dyes. This makes reliable multiple pathway tracing possible using fluorescent microscopy. Owing to the novelty of these AF conjugates of CTB, few studies have used them in relation to neuroanatomical pathway tracing. In this report, we describe a protocol that has been proved successful in the use of these new tracer conjugates to examine the neuroanatomical topography of thalamocortical connections[5]. Other studies have recently used these conjugates to examine connections in the peripheral nervous system[6–10]. Here we provide a formalized protocol of our technique using multiple fluorescent CTB conjugates to examine connections in the central nervous system. This technique has been tested in a large number of animals, within a pathway we have previously examined using dextran amines[11]. Many of the techniques discussed here can also be applied to other types of neuroanatomical tracers. Although this protocol focuses on the use of this technique in rats, the steps can be adapted for use in other species.

A common issue in most neuroanatomical studies is that fluorescent tracers are significantly less sensitive than their bright-field counterparts[12]. With AF-CTB, we consistently found highly sensitive retrograde labeling of connections. Of most importance, we found that due to the more sensitive nature of CTB, we were able to inject the tracer into a very confined injection site (sometimes <100 μm in width), yet still see extensive labeling patterns. Furthermore, the AF dyes proved to be exceptionally bright and photostable compared with the dyes attached to other neuroanatomical tracers[13]. The properties of these conjugates overcome many of the limitations of other fluorescent tracers.

Thus, this protocol adds to the current library of techniques for tracing multiple connections in the central nervous system. This protocol is most appropriate for experiments examining the difference in retrograde connections between multiple brain regions, and we suggest that AF-CTB should be the first-choice tracer for use in this type of experiment. Although this application is also feasible using multiple fluorescent conjugates of dextran amines, as reported by Reiner and Honig[14], the bi-directional transport of the dextrans and suboptimal fluorescent dyes conjugated to the dextrans cause many potential interpretation problems. Although it has been suggested that CTB also transports anterogradely, we found exclusively retrograde transport with this protocol. The nonfluorescent-based CTB tracing protocol by Angelucci *et al.*[15] (also see Ruigrok and Apps[16] and Dederen *et al.*[17]) should be used if a more permanent labeling technique is desired, because the most critical limitation of fluorescent tracers is the stability of the dye. Although the AF dyes are highly stable compared with conventional fluorescent dyes, they do fade slightly after extended illumination. We also suggest the use of this protocol when examining areas that require small, focal injection sites, because AF-CTB produces very bright and detailed labeling following small injection sites (<100 μm in width). However, although we include instructions for iontophoretic injections of CTB, we

have noticed suboptimal results when injecting AF-CTB with an iontophoretic technique, and we suggest caution for the investigator using this technique. Of course, as is the case with most neuroanatomical tracers, CTB does have the potential to be taken up by fibers of passage, although we noticed that this negative phenomenon was minimal[18]. CTB is compatible with most immunohistochemical techniques and other neuroanatomical tracers[15]. Because retrogradely transported CTB remains in vesicles and appears granular in cell somata, it does not display the detailed morphology of neuronal processes very well[19], although it is useful for experiments examining the size and shape of cell somata.

MATERIALS

REAGENTS

- Animals selected according to one's specific animal model (male Long–Evan Hooded rats, 325–350 g, approximately 12–16-weeks-old, from Harlan Laboratories) ! CAUTION The use of animals must conform to guidelines provided by relevant institutional animal care and use committees.
- AF conjugates of CTB (Invitrogen, cat. nos. C22841 and C22842) ▲ CRITICAL These are only available from Invitrogen. We cannot guarantee that the CTB conjugates from List Biological Laboratories will be compatible at the concentrations indicated in this protocol. Furthermore, we suggest that the 500-μm quantities of CTB should be ordered if possible, as the 100-μm quantities are difficult to handle.
- Phosphate buffer (0.06 M)
- Phosphate-buffered saline (PBS, 0.9% (wt/vol))
- Gelatin (1% (wt/vol)), from the porcine skin, type A (Sigma-Aldrich, cat. no. G2500-100G), for subbing slides
- Acetone (Fisher Scientific, cat. no. A18-4), for subbing slides
- Eukit mounting medium (Calibrated Instruments), for mounting cresyl violet sections
- Entellan mounting medium (EM Science, cat. no. 65037-71), for mounting fluorescent sections
- 70% (vol/vol) Ethanol (Fisher Scientific, cat. no. HC1000-1GL), 95% (vol/vol) Ethanol (Decon Labs, cat. no. 2801) and 100% (vol/vol) ethanol (Decon Labs, cat. no. 2701), for coverslipping
- Xylene (Fisher Scientific, cat. no. X3P-1GAL), for coverslipping
- Cresyl violet acetate (0.5% (wt/vol)) (Sigma-Aldrich, cat. no. C1791-5G), for staining cell bodies
- Paraformaldehyde (4% (wt/vol)) (Fisher Scientific, cat. no. T353-500) ! CAUTION A known carcinogen; wear gloves and mask.
- Sucrose fixative (30% (wt/vol) sucrose (Fisher Scientific, cat. no. S5-500) and 4% (wt/vol) paraformaldehyde), for cryoprotecting brain tissue
- 10% (vol/vol) povidone iodine
- Xylazine (Webster Veterinary)
- Ketamine (Webster Veterinary, cat. no. 07-805-8197)
- Isoflurane (Webster Veterinary, cat. no. 07-836-655)
- Beuthanasia D (pentobarbital solution, 1.4 ml dosage, Webster Veterinary, cat. no. 861565), or other appropriate euthanasia drugs.
- Lactated Ringer's solution (Webster Veterinary, cat. no. 13416)
- Ophthalmic base ointment (Webster Veterinary, cat. no. 450028)
- Gel foam (Henry Schein, cat. no. 9083300TV)
- Heparin, 100 USP (Henry Schein, cat. no. 110–5531)

EQUIPMENT

- Pipette puller (David Kopf Instruments, cat. no. Model 720)
- Glass pipettes (1.5 mm × 0.86 mm, non-filamented, A-M Systems, cat. no. 628000)
- Stereotaxic frame (David Kopf Instruments, cat. no. Model 900)
- Dissecting light microscope (Wild Heerbrugg, cat. no. M7A)
- Surgical instruments (hemostats, wound clips, microforceps, scalpel, scissors, spatula, etc.)
- Animal clippers (Fisher Scientific, cat. no. 01-305-10)
- Burr drill (Dremel: Model 275 type 5, with flex shaft 275-01 and drill bit #106)
- Germinator 500 (Fisher Scientific, cat. no. NC9956482)
- Iontophoretic device (Kation Scientific, cat. no. BAB-350)
- Picospritzer II (General Valve Corporation, cat. no. S1-302-900)
- Hamilton syringes (10 μl; Fisher Scientific, cat. no. 14-815-287)
- Freezing microtome (Lipshaw Microtome, model 80A; Stage: Physitemp BFS-30MP)
- Glass slides (Fisher Scientific, cat. no. 12-544-7)
- Coverglass (Fisher Scientific, cat. no. 12-548-5P)
- 24-well plates (Corning, cat. no. 3524)
- Cotton tip applicators (Fisher Scientific, cat. no. 23-400-101)
- 18G IV catheters (Webster Veterinary, cat. no. 720415)
- Microcentrifuge, 2,200*g* (Fisher Scientific, cat. no. 05-090-128)
- Fluorescent microscope (Zeiss Axioplan II, equipped with FITC (model 31001, Chroma Technology) and Texas Red (model 31004, Chroma Technology) filter sets)
- Spinning disk confocal microscope (DSU attached to IX81 microscope, Olympus)

REAGENT SETUP

Phosphate buffer To prepare 100 ml of phosphate buffer, mix 0.184 g of $NaH_2PO_4 \cdot H_2O$ (Fisher Scientific, cat. no. S369-1) and 1.43 g of Na_2HPO_4 (Fisher Scientific, cat. no. S374-500) into 100 ml of deionized (DI) water. Adjust pH to 7.0–7.4 with HCl or NaOH. Sterilize through a small pore filter into a sterile vile, and store at 4 °C.

PBS buffer To prepare 4 liters of PBS, mix 36.0 g of NaCl (Fisher Scientific, cat. no. S642-500), 7.36 g of $NaH_2PO_4 \cdot H_2O$ (Fisher Scientific, cat. no. S369-1) and 57.20 g of Na_2HPO_4 (Fisher Scientific, cat. no. S374-500) into 4 l of deionized (DI) water.

CTB solutions To prepare 0.5% (wt/vol) concentration, dissolve 500 μg of solid in 100 μl of phosphate buffer. To prepare 1% (wt/vol) concentration, dissolve 500 μg of solid in 50 μl of phosphate buffer. ▲ CRITICAL When mixing, do not vortex the solutions of CTB, as vortexing denatures CTB. Instead of vortexing, gently rotate the vial in the fingers until the solid is completely saturated.

Ketamine/xylazine cocktail For rats, the final solution of the cocktail should contain 87 mg/kg of ketamine and 13 mg/kg of xylazine. To prepare a 10-ml cocktail, mix 2.7 ml of ketamine, 1.5 ml of xylazine and 5.8 ml of sterile water. The dosage for rats is 0.1 ml/30 g per body weight.

PROCEDURE

Preparation of tracers ~30 min

1| Before ordering tracers, determine the amount of injections you would like to perform. Currently, four AF conjugates, a biotin conjugate and a horseradish peroxidase conjugate are available. The choice of the conjugate is dependent on the compatibility of microscope filters. For double injections (as we will discuss here), AF 488 and AF 594 CTB work the best. ■ PAUSE POINT The solid form of the tracers can be stored in the freezer (−20 °C) for about 6 months at a time. ? TROUBLESHOOTING

2| Prepare tracer solutions. For pressure injections, prepare a solution of tracers by diluting each conjugate separately in neutral phosphate buffer. For iontophoretic injections, several studies have reported using a slightly acidic buffer (pH 6.0) when using the non-fluorescent conjugates of CTB[3,4,18,20,21], although we have found satisfactory results with a neutral

buffer. The optimal concentration varies depending on the application, but we typically find that a 0.5–1% final working volume works well.
▲ CRITICAL STEP When mixing, do not vortex the solutions of CTB, as vortexing denatures CTB. Instead of vortexing, gently rotate the vial in the fingers until the solid is completely saturated.
? TROUBLESHOOTING

3| Quickly spin the solution in a centrifuge for 1 s to help the mixing and to move any solution residue to the bottom of the tube.
■ PAUSE POINT Solutions of CTB can be stored at 4 °C for about a week. For longer storage, separate the solid form into aliquots and store in the freezer (−20 °C) to avoid repeated freeze-thaw cycles when doing this. We do not recommend storing any solutions in the freezer, because we have noted reliability problems after using CTB that has been stored in the freezer in the solution form.
▲ CRITICAL STEP Protect this solution from light.
? TROUBLESHOOTING

Preparation for surgery ◷ TIMING ~30 min

4| Determine the coordinates for your area of interest using a stereotaxic atlas[22] (see **Fig. 1** for summary of surgical steps).

5| Determine method of tracer injection. For most cortical injections, we recommend one to use pressure injections using a picospritzer. Some studies use Hamilton syringes for pressure injections, but we have found that picospritzer injections are more effective because the tracer is injected in 'bursts' through a small pipette tip. For injections in subcortical areas, or for injections that require small injection sizes, iontophoresis through a pulsed constant current generator should be used. In this protocol, we focus on pressure injections using a picospritzer but also include brief instructions for iontophoretic injections.

6| Prepare the operating area by sterilizing equipments and by ensuring the tracers are prepared properly (**Fig. 1a**).

7| Before surgery, pull several capillary tubes in a micropipette puller (a 13.6-A current for the heating coil is used).

8| Immediately before a surgery, allow the tracer solutions to warm to room temperature (~20 °C) before injecting.

Surgery ◷ TIMING 1–4 h

9| Anesthetize the animal. In rats, we first place the animal in an induction chamber with isoflurane. Once the animal is sedated, an intraperitoneal injection of ketamine/xylazine cocktail is given (0.1 ml/30 g per body weight).

10| Once deep anesthesia is confirmed, shave the top of the animal's head and apply ophthalmic ointment on the eyes. Scrub the surgical site several times with 10% povidone iodine followed by 70% ethanol.

11| Place the animal into the stereotaxic device by placing blunt earbars into the ears (**Fig. 1b**; also see **Supplementary Video 1**). First, position a single bar into the right ear canal and tighten. Hold the animal in place and position the second earbar and tighten. If positioned correctly, one will not be able to move the animal's head side to side. It is very important to ensure that the earbars are secured into the ear canals properly, as pressure is placed on the skull throughout the procedure. Finally, place the animal's upper incisors into the mouth holder and secure them. Adjust the settings on the apparatus to ensure that the animal's head is in a level position.
▲ CRITICAL STEP The animal's head must be as secure and level as possible to ensure accurate injections.
▲ CRITICAL STEP All additional surgical steps should now be conducted under aseptic or sterile conditions, as is appropriate to the species, and under the guidelines of any relevant institutional animal care and use committees. At the very minimum, one is required to use a 'tips-only' technique where the sterility of the instrument tips must be maintained. However, we suggest that you use sterile surgical gloves and other simple aseptic techniques in addition to a 'tips-only' technique to provide an extra barrier of protection for the animal.
? TROUBLESHOOTING

12| Perform another scrub of the surgical site using povidone iodine and ethanol using aseptic conditions (use sterile surgical gloves). Drape the operating area using sterile towels.

13| Incise the skin sagittally along the sagittal suture (see **Supplementary Video 2**). Retract the skin away from the skull using clamps. Scrape the muscle and periosteum away from the skull using blunt-tipped instruments (**Fig. 1c**). We suggest that one should use sterile cotton-tipped applicators throughout the surgery to control bleeding. Continue dissecting and achieve hemostasis so that the skull surface is free of tissue and is dry (**Fig. 1d**). Bleeding from the bones of the skull can be alleviated using bone wax or a cautery.

Craniotomy

14| Place an empty pipette into the mount of the stereotaxic device. Move the pipette into position over the bregma and record the coordinates (**Fig. 1e**). Using predefined coordinates from an atlas[22], add or subtract the bregma coordinates and move the pipette to this position. Mark this position with a sharp pencil (**Fig. 1f**). Repeat this process for every injection site. If injection sites are close to the bregma, then 'create' a second bregma by moving the pipette to a different position and using this as a calibration point for the chosen coordinates.

15| Remove the pipette and perform a craniotomy using a Dremel drill (or an equivalent burr drill) with a round tip bit (**Fig. 1g–h**; also see **Supplementary Video 3**). Ideally, the burr holes should be made as small as possible, while allowing one to maneuver the micropipette. In the case of two injection sites that are less than about 5 mm apart, just drill one large burr hole. Otherwise, drill two separate burr holes and treat them as separate operating sites. If an injection site is <1 mm away from the sagittal suture, you should be very careful that the sagittal sinus is not punctured.

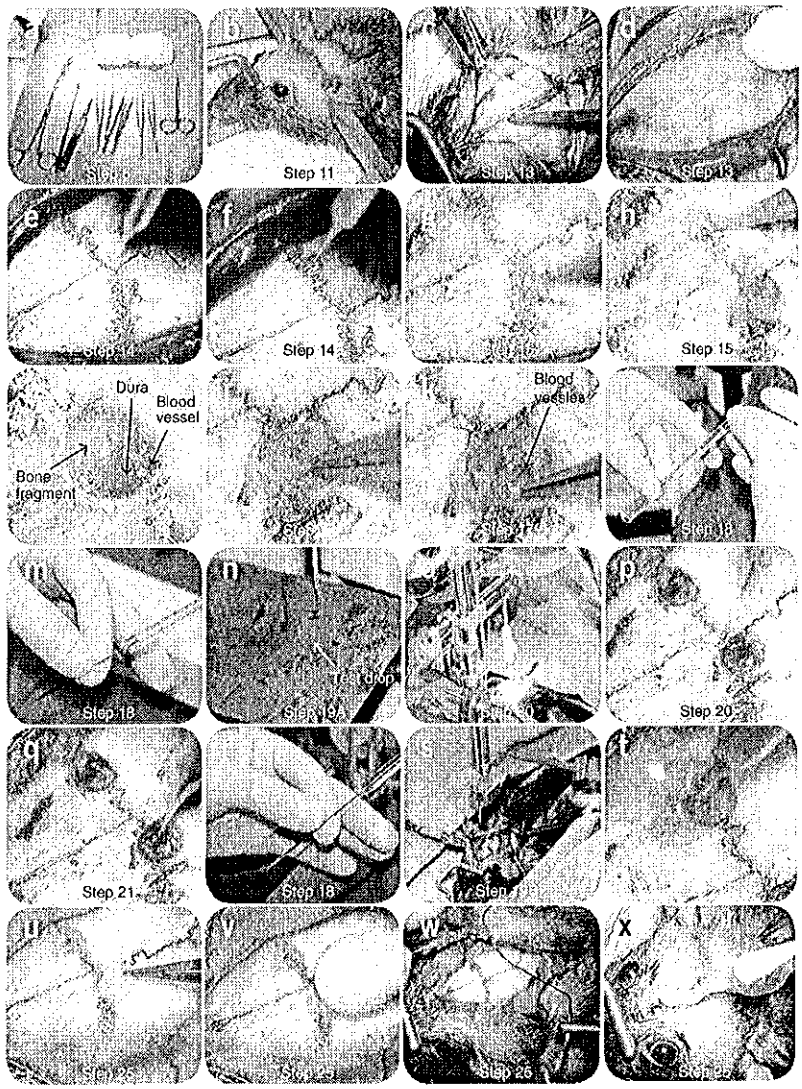

Figure 1 | Photographs of key surgical steps (a–x) from the PROCEDURE (Steps 9–25). Photographs were taken by a Leica M720 OH5 surgical microscope (courtesy of Bryan Thomasey). Permission to photograph the animal surgery was obtained from the University of Florida IACUC and Animal Care Services.

16| Throughout the process, use cotton swabs to remove any blood that has leaked into the burr hole. Furthermore, occasionally wipe the drill bit with an alcohol swab to remove any skull fragments. Continue drilling until the meninges is reached (**Fig. 1i**). At this point, you should expand the diameter of the burr hole so that you have more maneuverability and to create a buffer for the injection site in case you need to modify the coordinates slightly due to the presence of blood vessels.

⚠ CRITICAL STEP Avoid puncturing the brain surface or any blood vessels.

? TROUBLESHOOTING

17| Excise the dura mater using microforceps and a #11 scalpel blade tip and retract until the brain surface is exposed (**Fig. 1j** and **k**; also see **Supplementary Video 4**). Repeat this process for the other injection sites. If there are multiple burr holes, treat them as separate operating fields for the remainder of the procedure. Therefore, place wet sterile cotton swabs into each of the burr holes until you are ready to inject the tracers.

⚠ CRITICAL STEP If at any point you inadvertently rupture blood vessels on the surface of the brain, a good way to control the bleeding is to wet cotton swabs with water and use them to apply pressure to the brain surface. This will not completely stop the bleeding, but will decrease the severity. Alternatively, a bipolar cautery with microforceps can be used to cauterize any bleeding vessels.

⚠ CRITICAL STEP Depending on the drilling, some surgeries take quite a long time. If so, inject supplement doses of the anesthetic (10–20% initial dose) to maintain adequate anesthesia. However, be very conservative with the supplement doses to prevent any chance of overdosing the animal.

Tracer injections

18| Use a thin gauge Hamilton syringe to load the tracer into a micropipette (**Fig. 1l**). It is important to load the tracer without introducing air bubbles. Note that owing to the increased viscosity of CTB compared with other tracers (such as dextran amines), you cannot load the tracer through capillary action. In order to prevent air bubbles, load a minimum of 5 μl of tracer into the syringe. Place the syringe into the pipette until the point of the needle is at the bottom of the pipette. Slowly and evenly eject the tracer into the pipette from the syringe while slowly withdrawing the syringe needle (**Fig. 1m,r**). Once loaded, remove the syringe from the back of the pipette. Ensure that there are no air bubbles present that would interfere with the injections. After loading, quickly rinse the syringe with ethanol and sterile water before the tracer residue dries by tapping the barrel.
▲ CRITICAL STEP Use separate Hamilton syringes for each tracer conjugate in order to prevent any cross-contamination between the different conjugates.
? TROUBLESHOOTING

19| Mount the pipette into the apparatus. The procedure at this point differs slightly depending on if one is performing pressure (A) or iontophoretic (B) injections.

(A) Pressure injections

(i) Before injecting, determine the settings on the picospritzer. With a picospritzer, the volume injected is dependent on the pipette tip, p.s.i. of gas and injection time. We calculate these settings based on the visual size of a drop created by the settings. To determine these settings, we do several test injections onto a piece of X-ray film, so that we can see how large the drops are. Ideally, one should adjust the tip and picospritzer settings to create the smallest-sized drop that will exit the pipette tip (**Fig. 1n**). If the settings create too small of a drop, the liquid will not clear the pipette tip and will instead cling onto the glass. Usually, we use about 20 p.s.i. with 10–15 ms injection duration and then adjust the pipette tip using microforceps until we see our desired drop size. This means that the picospritzer uses a pressure of 20 p.s.i. to inject for a 10–15 ms long 'burst.' We usually use about 10 bursts over the course of an injection. Of course, if one wants to inject into a small area, one should use a smaller pipette tip.

(B) Iontophoretic injections

(i) If performing iontophoretic injections, place one of the leads into the pipette and ensure that it is immersed in the tracer. Place the grounding lead onto the animal's skin (**Fig. 1s**).
? TROUBLESHOOTING

20| Position the pipette over bregma and recalibrate with the atlas coordinates (**Fig. 1o,p**). Then, move the pipette to the injection site and ensure that it will not hit any major blood vessels when lowered into the brain. If there are any impediments at the injection site, adjust the injection coordinates slightly such as to avoid the vessels. If blood enters the pipette tip, you can remove the blood by ejecting some of the tracer using the picospritzer.
▲ CRITICAL STEP If you accidentally hit the pipette in any way, move the arm of the apparatus or adjust the pipette tip diameter after calibrating, you must recalibrate the coordinates. This is to insure the accuracy of the bregma calibrations.

21| When ready to inject, move the pipette into position until it touches the brain surface and read the dorsal–ventral coordinate (**Fig. 1q**). Slowly lower the pipette into the brain until the desired depth is reached. Sometimes, the pipette tip does not easily pierce the surface due to the pia mater. If this is the case, then carefully make a small incision over the injection site.
▲ CRITICAL STEP If performing iontophoretic injections, always use a constant negative current (−0.5 μA) when moving the pipette through the brain. This helps to minimize any unwanted injection track from appearing when moving the pipette through the brain.

22| Begin injecting the tracer.

(A) Picospritzer injections

(i) When injecting, always look at the pipette and ensure that the meniscus moves with each injection burst. Sometimes the pipette tip will clog with blood on its way down to the injection site. If this happens, increase the duration of the picospritzer until the meniscus moves and then change back to the original settings.

(ii) Inject the smallest amount possible, but enough to see adequate labeling. The amount to inject will depend on the experiment and will take experience to optimize. Normally, with the settings mentioned above, we use about 10 bursts for cortical injections. Wait about 10 s between each burst.

(B) Iontophoretic injections

(i) When ready to inject, use positive 4 μA pulses (on/off in 5-s intervals) for 30–60 min (the specific voltage and timing vary depending on the experiment) (**Fig. 1t**).

(ii) When removing the pipette from the brain, switch back to a continuous negative current (−0.5 μA).
▲ CRITICAL STEP Regardless of the injection method, we have found that removing the pipette at a very slow pace (1 min to completely move the pipette from the injection to the surface of the brain) usually minimizes the injection track. If the pipette is withdrawn too quickly, we believe that negative pressure caused by the tracer injection will force some of the tracer along the injection track of the pipette. Some studies have also reported that leaving the pipette at the injection site for about 15 min prevents leakage of the tracer[16,23], although we have not noticed any significant difference when doing this.
? TROUBLESHOOTING

23| Once the pipette has been removed, quickly rinse the surface of the brain with sterile water to prevent any leaked tracer to be absorbed by layer I of the cortex.

24| Repeat Steps 18–23 for the other injection sites as applicable (**Fig. 1r–t**). Use new pipettes for each additional injection.

25| Once all injections are finished, thoroughly rinse the skull surface with sterile water and ensure that all pencil markings are removed (see **Supplementary Video 5**). Place sterile gel foam into each burr hole (**Fig. 1u,v**). Suture the wound and apply antibiotic ointment (**Fig. 1w** and **x**). Inject 5 ml of Ringer's lactate solution intraperitoneally and place the animal in a warm recovery area.

Perfusion ◷ TIMING **1–2 h**

26| Prepare reagents and materials for the perfusion. Approximately 1 liter of 4% paraformaldehyde and heparinized PBS (use about 5 ml of 100 USP heparin per liter of PBS) is required. Heat the heparinized PBS to physiological temperature (37 °C).

27| After a survival time of typically 7 d, inject the animal with Beuthanasia-D (or other pentobarbital solution) and open the thoracic cavity to access the heart and aorta (transcardial perfusion technique). We found that it is easiest to insert an intravenous catheter (as opposed to a needle) into the left ventricle and then move the catheter into the aorta through the aortic valve.
⚠ CRITICAL STEP It is vital to begin the perfusion while the heart is still beating in order to ensure that there is no clotting. Any blood left in the brain will autofluoresce under the fluorescent microscope.

28| During the procedure, first perfuse with heparinized saline at physiological temperature (37 °C). Once the blood exiting the heart is clear (or ~0.5 ml of saline is used), perfuse with 4% paraformaldehyde. Continue the fixative perfusion until the flow of the liquid stops, or use ~0.5 liter of fixative.
? TROUBLESHOOTING

29| After the perfusion, remove the brain and place in a postfix solution.
⚠ CRITICAL STEP If the brain will be cut using a frozen sectioning procedure, place the extracted brain into the postfix solution of 30% sucrose in 4% paraformaldehyde for 3 d (or until the brain 'sinks' in the solution) at 4 °C to cryoprotect the cells.
⏸ PAUSE POINT You can store the brain for several days in 4% paraformaldehyde at 4 °C. It is recommended that the brain should not be stored this way after it has already been cryoprotected in the sucrose/paraformaldehyde solution.

Histology ◷ TIMING **3–4 h plus drying time**

30| Cut the brain using the desired method, once cryoprotection is complete. We have seen good and consistent results using a freezing stage sliding microtome system. In our experiments, we cut the brain into 40-μm sections.

31| Mount the sections serially onto glass slides. For CTB cases, we mount two series of fluorescent sections and one cresyl violet series in order to assist in identifying brain regions. When dry, coverslip fluorescent sections with Entellan and coverslip cresyl violet sections with Eukit (see **Boxes 1** and **2** for more information about cell processing).
⚠ CRITICAL STEP Although Entellan protects the sections from photobleaching, always protect any fluorescent sections from light.
⏸ PAUSE POINT Sections should be stored at room temperature in the dark. Although the AF conjugates are very stable, we recommend to examine the fluorescent sections as soon as possible in order to avoid issues arising from fluorescence fading.
? TROUBLESHOOTING

BOX 1 | SUBBING SLIDES ◷ TIMING 1–2 H PLUS DRYING TIME

1. Prepare 1% gelatin solution by adding the gelatin powder into water and heating until dissolved.
⚠ CRITICAL STEP Do not boil the gelatin solution.
2. Load several non-charged glass slides into metal or glass trays.
3. Dip trays several times in each of the following solutions in the following order (ensure to drain trays before placing in next solution):
   (i) 50% acetone;
   (ii) DI water rinse;
   (iii) 70% ethanol;
   (iv) 95% ethanol;
   (v) 95% HCl;
   (vi) 95% ethanol;
   (vii) 70% ethanol;
   (viii) DI water;
   (ix) Warm subbing solution (1% gelatin).
⚠ CRITICAL STEP The solution should be slightly warm in order to prevent the gelatin from solidifying.
4. Place on absorbent cover and allow to dry. Be sure to cover the trays to avoid dust accumulation.

BOX 2 | CELL PROCESSING ● TIMING 2 D

1. Cut 40-μm-thick serial brain sections using a freezing stage sliding microtome. Place into 24-well tissue culture plates in PBS.
2. Mount each series onto gelatin-subbed slides.
3. Let dry overnight, flat, in the dark.
4. Process the slides based on the desired staining method. In each method, dip the slides several times (4–5 times) in respective staining dishes containing particular reagents. In each submersion, unless indicated otherwise, the slides should be submersed several times until any residue from the preceding step is fully rinsed off. Generally, each step will involve several submersions of the slides, about 30–60 s total.

(A) Fluorescent sections

(i) 70% ethanol
(ii) 95% ethanol
(iii) 100% ethanol
(iv) 100% ethanol
(v) Xylene for about 3–5 min

(B) Cresyl violet

(i) 70% ethanol
(ii) 95% ethanol
(iii) 100% ethanol
(iv) 100% ethanol
(v) Xylene for about 3–5 min
(vi) 100% ethanol
(vii) 100% ethanol
(viii) 95% ethanol
(ix) 70% ethanol
(x) DI water
(xi) 1% Cresyl violet solution. The amount of time in this solution varies from 30 to 60 s. Adjust as needed to achieve optimal staining.
(xii) DI water
(xiii) 70% ethanol
(xiv) 95% ethanol
(xv) Differentiate in 95% ethanol and observe under a microscope for best contrast. Neurons should appear with a dark staining nucleolus surrounded by a clear nucleoplasm. Extracellular background should be white or very pale blue.
(xvi) 100% ethanol
(xvii) 100% ethanol
(xviii) Xylene for about 3–5 min
(xix) Xylene for about 3–5 min

⚠ CRITICAL STEP Do not allow the slides to dry out between each step. The slides should be moved to the next solution immediately.

5. Coverslip with Entellan for fluorescent sections and with Eukitt for cresyl violet. Note that these are xylene-based adhesives; therefore, this procedure is not compatible with water-based adhesives. Avoid introduction of air bubbles.
6. Allow to dry flat at least 24 h.

32| View sections under a fluorescent microscope using a compatible filter set. We use a FITC and Texas red filter set to visualize AF 488 and AF 594, respectively.

? TROUBLESHOOTING

● TIMING

Preparation of tracers (Steps 1–3): ~30 min
Preparation for surgery (Steps 4–8): ~30 min
Surgical procedures (Steps 9–25): 1–4 h
(survival time ~7 d)
Perfusion (Steps 26–29): 1–2 h
(post-fix incubation time (Step 29): 3 d)
Histology (Steps 30–32): 3–4 h plus drying time
Subbing slides (**Box 1**): 1–2 h plus drying time
Cell processing (**Box 2**): 2 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

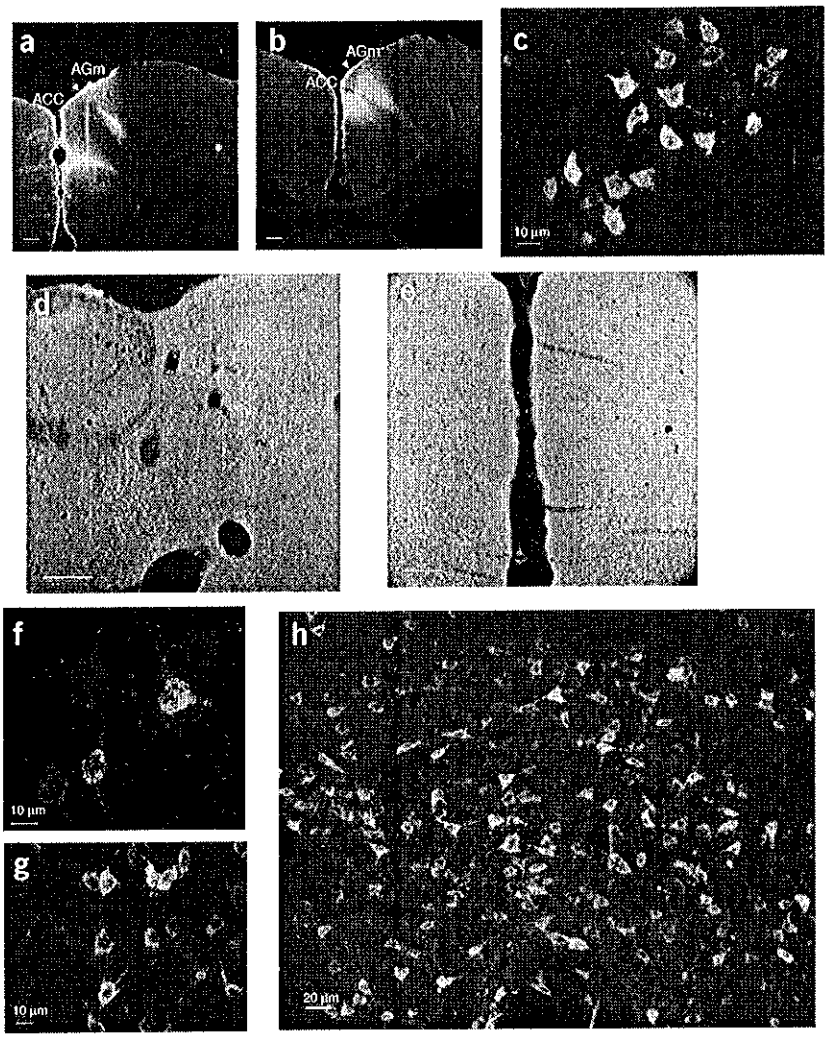

Figure 2 | Example injection sites and labeling patterns of AF 488 (green) and 594 (red) CTB. Pictures containing multiple conjugates were taken separately under their respective filter set (FITC for AF 488 and Texas Red for AF 594) and merged together into one image. (**a**) Sample case with injection sites placed in different cortical areas. AF 488 CTB was injected into the anterior cingulate cortex (ACC) and AF 594 CTB was injected into the medial agranular cortex (AGm). Note that owing to the proximity of our injection sites to the brain surface, some of the green conjugate leaked out and was absorbed by cortical layer I during the injection. An injection track is also present on the green injection site. (**b**) Sample case with adjacent injection sites (same intended brain regions as panel **a**). (**c**) High-magnification confocal image of labeling patterns in the lateral posterior thalamus (LP) from the injection site in panel **a**. The labeling appears granular in the somata and is focused on the periphery of the cell membrane. Several of the cells in this field of view are double-labeled (yellow in appearance). Often, the two conjugates were located in different areas of a single cell. (**d**) Sample labeling pattern in the thalamus from a case with injection sites in similar regions as panels **a** and **b**. Note the yellow appearance of several double-labeled cells. (**e**) Sample cortical labeling pattern (the brain midline is in the center of the image view) following bilateral injection of the two conjugates into the striatum. (**f**) High-magnification confocal image of AF 594 CTB labeling. (**g**) High-magnification confocal image of AF 488 CTB labeling. (**h**) Confocal image of labeling in the LP. The cells are granular in appearance, and several double-labeled cells are present. There is also some proximal dendritic labeling. Bars, 200 μm, unless otherwise noted. Panel **a** adapted with permission of the publisher[5]. Experiments were conducted under the supervision of the University of Florida IACUC.

ANTICIPATED RESULTS

A successful experiment should show a discrete injection site and well-defined labeled cells (**Fig. 2**). The main advantage to AF-CTB is the ability to have highly focused and narrow injection sites. Double injections of two conjugates can be given very close to each other (**Fig. 2a,b**). Labeling patterns using multiple conjugates should appear very clear and defined (**Fig. 2c–h**). At high magnification, transported CTB should appear granular in cells, with some proximal dendritic labeling (**Fig. 2c,f** and **g**). The granular appearance is due to the fact that transported CTB remains in vesicles[19]. The nuclei of labeled cells are unstained and the staining is focused on the plasma membrane (**Fig. 2c,f–h**). When cells are doubled-labeled by two conjugates of CTB, the different conjugates appear to be located in different parts of the cell (**Fig. 2c**).

There has been some debate in the scientific community about whether CTB is an anterograde or retrograde tracer[15,20]. In our experience with this protocol applied to forebrain connections, we have found that it is mostly a retrograde tracer. However, we tend to see a small amount of anterograde labeling if there is observable tissue damage at the injection site. This may be due to uptake by damaged neurons.

In summary, fluorescent tract-tracing using CTB is a vital tool that allows investigators to examine and compare results from multiple injection sites in the same animal. The stable nature of the AF dyes overcomes two principal challenges involved in using fluorescent tracers, brightness and photostability.

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS We greatly thank Maggie Stoll for her continued technical help. We also thank the help of Dr. Stephen B. Lewis, Robin Barry and Bryan Thomasey for providing equipment for the video clips. We also appreciate further technical help from Elyse Morin and Rachel Tai. This study was supported in part by the NIMH Grant MH60399.

Published online at http://www.natureprotocols.com.
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions.

1. Krout, K.E., Belzer, R.E. & Loewy, A.D. Brainstem projections to midline and intralaminar thalamic nuclei of the rat. *J. Comp. Neurol.* **448**, 53–101 (2002).
2. Llewellyn-Smith, I.J., Martin, C.L., Arnolda, L.F. & Minson, J.B. Tracer-toxins: cholera toxin B-saporin as a model. *J. Neurosc. Methods* **103**, 83–90 (2000).
3. Luppi, P.-H., Fort, P. & Jouvet, M. Iontophoretic application of unconjugated cholera toxin B subunit (CTb) combined with immunohistochemistry of neurochemical substances: a method for transmitter identification of retrogradely labeled neurons. *Brain Res.* **534**, 209–224 (1990).
4. Zin-Ka-Ieu, S., Roger, M. & Arnault, P. Direct contacts between fibers from the ventrolateral thalamic nucleus and frontal cortical neurons projecting to the striatum: a light-microscopy study in the rat. *Anat. Embryol.* **197**, 77–87 (1997).
5. Conte, W.L., Kamishina, H., Corwin, J.V. & Reep, R.L. Topography in the projections of lateral posterior thalamus with cingulate and medial agranular cortex in relation to circuitry for directed attention and neglect. *Brain Res.* **1240**, 87–95 (2008).
6. Christianson, J.A. *et al.* Convergence of bladder and colon sensory innervation occurs at the primary afferent level. *Pain* **128**, 235–243 (2007).
7. Kreier, F. *et al.* Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes. *Endocrinology* **147**, 1140–1147 (2006).
8. McDavid, S., Lund, J.P., Auclair, F. & Kolta, A. Morphological and immunohistochemical characterization of interneurons within the rat trigeminal motor nucleus. *Neuroscience* **139**, 1049–1059 (2006).
9. Niedringhaus, M. *et al.* Brainstem sites controlling the lower esophageal sphincter and crural diaphragm in the ferret: a neuroanatomical study. *Auton. Neurosci.* **144**, 50–60 (2008).
10. O'Malley, M.R. *et al.* Fluorescent retrograde axonal tracing of the facial nerve. *Laryngoscope* **116**, 1792–1797 (2006).
11. Kamishina, H. *et al.* Cortical connections of the rat lateral posterior thalamic nucleus. *Brain Res.* **1264**, 39–56 (2009).
12. Schofield, B.R., Schofield, R.M., Sorensen, K.A. & Motts, S.D. On the use of retrograde tracers for identification of axon collaterals with multiple fluorescent retrograde tracers. *Neuroscience* **146**, 773–783 (2007).
13. Panchuk-Voloshina, N. *et al.* Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J. Histochem. Cytochem.* **47**, 1179–1188 (1999).
14. Reiner, A. & Honig, M.G. Dextran amines: versatile tools for anterograde and retrograde studies of nervous system connectivity. In *Neuroanatomical Tract-Tracing 3* (eds. Zaborszky, L., Wouterlood, F.G., & Lanciego, J.L.) 304–335 (Springer Science+Business Media Inc., New York, 2006).
15. Angelucci, A., Clascá, F. & Sur, M. Anterograde axonal tracing with the subunit B of cholera toxin: a highly sensitive immunohistochemical protocol for revealing fine axonal morphology in adult and neonatal brains. *J. Neurosc. Methods* **65**, 101–112 (1996).
16. Ruigrok, T.J.H. & Apps, R. A light microscope-based double retrograde tracer strategy to chart central neuronal connections. *Nat. Protoc.* **2**, 1869–1878 (2007).
17. Dederen, P., Gribnau, A. & Curfs, M. Retrograde neuronal tracing with cholera toxin B subunit: comparison of three different visualization methods. *Histochem. J.* **26**, 856–862 (1994).
18. Chen, S. & Aston-Jones, G. Evidence that cholera toxin B subunit (CTb) can be avidly taken up and transported by fibers of passage. *Brain Res.* **674**, 107–111 (1995).
19. Kobbert, C. *et al.* Current concepts in neuroanatomical tracing. *Prog. Neurobiol.* **62**, 327–351 (2000).
20. Datiche, F. & Cattarelli, M. Reciprocal and topographic connections between the piriform and prefrontal cortices in the rat: a tracing study using the B subunit of the cholera toxin. *Brain Res. Bull.* **41**, 391–398 (1996).
21. Tsumori, T., Yokota, S., Ono, K. & Yasui, Y. Organization of projections from the medial agranular cortex to the superior colliculus in the rat: a study using anterograde and retrograde tracing methods. *Brain Res.* **903**, 168–176 (2001).
22. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates* (Elsevier Academic Press, San Diego, 2005).
23. Apps, R. & Ruigrok, T.J.H. A fluorescence-based double retrograde tracer strategy for charting central neuronal connections. *Nat. Protoc.* **2**, 1862–1868 (2007).