



Published in final edited form as:

Neurol Res. 2016 July ; 38(7): 561–569. doi:10.1080/01616412.2016.1153820.

## Histological Methods for *ex vivo* Axon Tracing: A Systematic Review

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### Abstract

**Objectives**—Axon tracers provide crucial insight into the development, connectivity, and function of neural pathways. A tracer can be characterized as a substance that allows for the visualization of a neuronal pathway. Axon tracers have previously been used exclusively with *in vivo* studies; however, newer methods of axon tracing can be applied to *ex vivo* studies. *Ex vivo* studies involve the examination of cells or tissues retrieved from an organism. These post mortem methods of axon tracing offer several advantages, such as reaching inaccessible tissues and avoiding survival surgeries.

**Methods**—In order to evaluate the quality of the *ex vivo* tracing methods, we performed a systematic review of various experimental and comparison studies to discern the optimal method of axon tracing.

**Results**—The most prominent methods for *ex vivo* tracing involve enzymatic techniques or various dyes. It appears that there are a variety of techniques and conditions that tend to give better fluorescent character, clarity, and distance traveled in the neuronal pathway. We found direct comparison studies that looked at variables such as the type of tracer, time required, effect of temperature, and presence of calcium, however, there are other variables that have not been compared directly.

**Discussion**—We conclude there are a variety of promising tracing methods available depending on the experimental goals of the researcher, however, more direct comparison studies are needed to affirm the optimal method.

### Keywords

Carbocyanine; Axon tracing; Axon labeling; DiI; NeuroVue; Ex vivo; Postmortem; Fixed tissue

### Introduction

Axon tracing methods are invaluable for studying the anatomy of neuronal pathways in normal and pathological states, but the most commonly used method, *in vivo* application of a tracer, requires survival surgery which causes a risk of research animal morbidity or discomfort.<sup>1,2,3</sup> Chen (2006) suggests that the risk for animal loss associated with an additional surgery, and thereby valuable data, is 10–20 percent.<sup>4</sup> Other drawbacks include

the fact that the target pathway is often difficult to reach or inaccessible.<sup>2</sup> Fortunately, within the last few decades, substantial advancements have been established regarding techniques to study neuronal pathways *ex vivo* by removing organs, or parts of organs, and subsequently applying a tracer. *Ex vivo* studies have many characteristics that make them advantageous in studying neuroanatomy. For instance, *ex vivo* application is beneficial because it eliminates the need to perform a survival surgical procedure for tracer application. Additionally, it is possible to trace tissues that would otherwise be inaccessible *in vivo*.

The most commonly used methods for axon tracing *ex vivo* use various carbocyanines dyes. These dyes are capable of being use in *ex vivo* studies due to their lipophilic nature. Upon application to fixed tissues, the dyes possess the ability to diffuse through the fixed plasma membranes of neurons.<sup>4</sup> Axon tracers exhibit the potential to trace neuronal pathways anterograde (away from the cell body), retrograde (towards the cell body), or bidirectionally.<sup>4</sup> Illustrations demonstrating the directionality of axon tracers are shown in figure 1 and 2. Once in the plasma membrane, carbocyanines have been shown to move bidirectionally and even to adjacent cells through the aqueous medium.<sup>2,3,4,5</sup> Carbocyanine dyes are fluorescent, making visualizing morphology and connectivity along a desired axonal pathway feasible.<sup>4</sup> Figure 3 illustrates the various colors emitted at the corresponding wavelength by different carbocyanines. Due to the different colors emitted, multiple carbocyanines can be used for two-color or three-color tracing. The most frequently used carbocyanines are 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), 4-4-dihexadecylaminostyryl (DiA), and 3,3'-9-dioctadecyloxacarbocyanine perchlorate (DiO).<sup>2,4</sup> Other viable methods for axon tracing include other lipophilic dyes, dextrans, and multiple pigments, including, Horseradish Peroxidase (HRP), Lucifer Yellow, Mini Ruby, and NeuroVue Dyes.<sup>1,3,6,7,8,9</sup> We sought to compare the available methods and refinements for axon tracing in order to determine which histological method is the most effective for *ex vivo* axon tracing.

## Methods

We searched Pubmed in December 2015 using the search terms: (fixed OR postmortem OR *ex vivo*) AND ((neur\* OR axon\*) AND trac\*). We included full-text articles in English published prior to December 2015 that contained unique experimental data for *ex vivo* placement of an axon tracer. We also reviewed the references of our selected articles for additional studies that matched our selection criteria.

## Results

Our search resulted in 275 articles, of which 14 matched our inclusion criteria. We found twelve other matching studies by reviewing references in our selected articles, making a total of 26 articles. These studies are summarized in Table 1. Table 2 contains the major conclusions we drew based on the studies that incorporated comparative data. Although all the studies indicated the usage of a tracer, there was variability among likely key variables of the method. Aspects of the methods that may be of importance for precise and clear tracing include the species evaluated, location of tracer insertion, fixation method, incubation

solution, temperature, duration, tissue sectioning method, histology method, visualization method, and method for quantification.

From analyzing Table 2, it is apparent that the most frequently used tracer is DiI. Twenty out of the 23 studies referenced in Table 2 use some form of DiI as the preferred axon tracer. This can be attributed to the fact that there are a limited number of studies that use a new tracer. For example, since NeuroVue dyes are more recent relative to the carbocyanine dyes, there are fewer studies to assess the performance of this tracer. However, it is consistently seen that among the carbocyanine dyes, DiI is the preferred tracer. In regards to the form of the tracer, DiI is available in a crystallized form as well as an oil-based form. The Murphy and Fox (2007) study suggests that DiI in the crystallized form obtains slightly faster rates of diffusion as well as increased staining quality.<sup>3</sup> This is reflected in the fact that 20 out of 21 studies in Table 1 that used DiI used it in its crystallized form. NeuroVue dyes appear to work by similar mechanisms to the carbocyanine dyes. Similarly, these NeuroVue dyes are lipophilic – they are capable of traveling through the aldehyde fixed membranes of cells by lateral diffusion. Additionally, both DiI and NeuroVue dyes are compatible with counterstaining and immunohistochemical techniques, therefore making these carbocyanines advantageous. However, it is crucial to be wary of the detergents used during immunohistochemical methods.<sup>8</sup> Some stronger detergents such as Triton X may be too harsh to use with DiI and will promote dye leakage out of the cells; therefore, Tween 20 may be compatible with axon tracing methods.<sup>2</sup>

An apparent advantage to the carbocyanine tracers is that they appear to work consistently in a variety of tissue types. Chen *et al* (2006) suggests that the carbocyanine dyes do not discriminate between human and guinea pig tissue or between peripheral and central nervous system tissue.<sup>4</sup> Therefore, this method seems to work well with a variety of species. On the contrary, data suggests that although dextran methods are excellent for labeling adult neural connections, they may have limited usefulness in embryos and afferent cells.<sup>8,10</sup>

There is a diversity of tracer application methods among different studies. The DiI crystals are most frequently dissolved in organic solvents and then dried onto a micropipette tip. Once the pipette tip is dry, the crystals can be dabbed at the site of interest. However, other plausible methods exist for tracer insertion, such as using pins, injections, and dye-impregnated filters.

Four percent paraformaldehyde (PFA) tends to be the standard fixation solution in 19 out of the 26 studies. Additionally, four percent PFA also was a prominent choice for the incubation solution. Chen *et al* (2007) compares two different PFA concentrations (one percent and four percent).<sup>4</sup> The study results suggest that there is no significant difference between the two; therefore, it appears that the investigator can choose the concentration of the PFA based on preference. Occasionally, studies such as Hoffman (1998) integrated 0.1 percent ethylenediaminetetraacetic acid (EDTA) into the incubation solution to aid in decalcification of the tissue.<sup>11</sup>

Substantial variability exists in the incubation temperatures; these temperatures ranged from 6° C to 40° C. Colder temperatures limit diffusion, while higher temperatures promote

diffusion. However, as a consequence of higher temperatures, the amount of non-specific staining may increase as a result of dye leakage out of the membranes.

The use of a vibratome appears to be the preferred method of sectioning, found in 19 of 27 studies. However, the studies did not always explicitly state the sectioning method. One study announced that there was a potential for the cryostat to promote dye leakage by destabilizing the dye.<sup>2</sup>

## Discussion

Successful histological methods for axon tracing in *ex vivo* tissue have the potential to aid in a better understanding of neuron behavior, connectivity, and treatments for various pathological states.<sup>2,3,4,12</sup> After review of the selected articles, it is apparent that there are both advantages and disadvantages associated with each method. Thus, the best method varies depending on the research goals of the user. Each researcher may have different interpretations as to what constitutes the best method or axon traced tissues. For example, these differences in interpretation may be in regards to ease of use, cost, distance, time, and sharpness. Although direct comparison studies are the most helpful for providing valuable information regarding factors that affect the effectiveness of a tracer, the majority of our included studies only used a single method. Consequently, our ability to draw conclusions about superiority over other methods was limited. Table 2 summarizes the major findings and conclusions drawn from the seven comparison studies we obtained from our search. Trends among studies were apparent and will be outlined in the subsequent paragraphs. From all of the selected articles, it is evident that *ex vivo* tracing methods originally used Lucifer Yellow and HRP in the 1980s.<sup>1,6,13,14</sup> Since then, tracers started to deviate from HRP and Lucifer Yellow to using the long distance, lipophilic dyes such as the carbocyanines. Carbocyanines are the most frequent method used. NeuroVue dyes are suggested to be promising according to the two direct comparison studies.<sup>7,10</sup> We will outline this progression of tracers and provide potential explanations for the observed trends.

Lucifer Yellow is common in studies from the 1980s and 1990s. Although still used, its prevalence has dwindled due to the development of other *ex vivo* tracer counterparts. While this tracer has the potential for labeling a single neuron structure, it lacks the capability to trace long distances. This method requires injecting the individual neuron iontophoretically in four percent PFA fixed tissues. Therefore, injecting many neurons can be time consuming (>50 min) and require precision.<sup>6</sup> Consequently, this technique does not require lengthy incubation periods compared to other tracers such as the carbocyanines. This method also works with storing the tissue in the cold, whereas other lipophilic tracers require room temperature or above for diffusion. Many of the studies had the sections cut with a vibratome after being treated with gelatin for support during the sectioning process. Another feature that emerged while analyzing studies is that Lucifer Yellow's characteristic fluorescent color diminishes due to exposure of illumination after a couple of weeks.<sup>14</sup> Applying the DAB method allows for the fluorescent neuron to convert to a dark color, which aids in a longer stabilization of the labeling. DAB processed tissue can be viewed under an electron microscope. While DAB aids in overcoming the issue with the stability of

the fluorescent neuron, it also has the potential to hinder visualization of precise neuronal processes.<sup>6</sup>

Similar to Lucifer Yellow, HRP was established in the mid-1980s. This technique requires the tracer to be injected with a micropipette under the visual guidance of a stereo microscope. The duration of injection was recorded to be five to 15 minutes (Haber 1988 and Kageyama 1987). This technique also requires precision for insertion. Incubation solutions in different studies showed variability. Haber *et al* reported longer distances achieved using a low calcium phosphate buffer; however, it is difficult to discern what exactly is responsible for these differences in distance.<sup>13</sup> Variation was also noted in regards to incubation duration, making it difficult to form a conclusion discerning the best tracing method. In both studies, HRP methods use a DAB procedure in order to view the neurons with an electron microscope. Kageyama *et al* indicates that disadvantages of this tracing method include “the process of injection or the mere presence of the marker itself may cause tissue damage or may alter the structure, activity or behavior of cells.”<sup>1</sup> Different studies reported a range of distances. For instance, Kageyama reported that the tracer diffused only 1.5 mm from the injection site, while Haber 1988 reported 10 mm.<sup>1,13</sup> The differences may be due to Haber *et al* using Wheat Germ Agglutinin -HRP while Kageyama used generic HRP.<sup>1,13</sup> Without a direct comparison study to assess these differences in methods, the variables responsible for these differences cannot be precisely determined.

DiI is suggested to be the most frequently used method for axon tracing among the carbocyanines. Due to other comparison studies, Chen *et al* (2006), Lukas *et al*, (1998) and Fritsch (2005) reiterated that DiI advances through the tissue more quickly than the other carbocyanines.<sup>2,4,7</sup> The rate of diffusion for DiI seemed to be similar among studies conducted independently. Lukas *et al* (1998) identified a diffusion coefficient to be  $1.14 \times 10^{-2}$  mm/hr and similarly, Swift *et al* (2004) found the control to have a rate of diffusion of  $1.20 \pm 0.08 \times 10^{-2}$  mm/h.<sup>2,15</sup> DiI travels not only farther and faster, but it also continues to travel even after several weeks, whereas DiO ceases to diffuse after a couple of weeks. Fritsch *et al* (2005) stated that “DiO is impractical as a long range tracer” and has “limited usefulness”.<sup>7</sup> Additionally, DiI is more favorable because it exhibits more fluorescent character than other dyes. An aspect that is found to increase the efficiency of the DiI tracer is applying DiI in crystallized form.<sup>3</sup> Murphy *et al* (2007) stated that DiI in oil form takes a week longer than crystallized DiI to diffuse.<sup>3</sup>

Despite these differences, there are certain trends towards agreement between authors of the included articles. First, it is common for 0.1 percent EDTA to be added to assist in binding to the excess calcium in the tissue, reducing the occurrence of transneuronal labeling.<sup>3,11</sup> Second, temperature assists in increasing the rate of diffusion of the dye; however, it can also facilitate the tracer to diffuse out of the neuron.<sup>2,4,11</sup> We found that it is the most common to allow the tissue to incubate in four percent PFA in 0.1M phosphate buffer (PB). A vibratome is suggested to be the best option for sectioning tissue embedded in gelatin.<sup>1,2,4,6,7,11,12,15</sup> Cryoprotected tissues sectioned on a microtome may promote dye leakage.<sup>2</sup>

Discrepancies arise with the method of insertion and incubation period for many of the studies. Most frequently, the tool used to insert the dye was a glass micropipette. However, many other techniques were successful, including pins and dye-covered filters. There are too many changing variables between studies to discern which method for inserting the dye achieves the most desirable results. Even studies using the same tool may insert the dye in a different way. For example, Richards *et al* (1997) inserted the tracer via micropipette through the length of the tissue and dispensed the tracer every few millimeters.<sup>12</sup> Other methods that also used a micropipette involved placing DiI in the tissue superficially. Both techniques seem to be feasible. In regards to incubation period, the studies all came to a consensus that at some point, the tracer would cease to diffuse. However, the time (measured in weeks) among the studies differed substantially. Some studies such as Lukas *et al* (1998) indicated that the diffusion of the dye plateaued at 12 to 15 weeks.<sup>2</sup> However, other studies like Chen *et al* (2006) specify that the dye continued to diffuse from nine to 24 weeks at a decreased rate.<sup>4</sup> Other studies maintained the tracer diffusion for a couple of days to a few weeks. In some cases, it was even reported that 24 hours was sufficient for dye incubation.<sup>15</sup> Temperature, varying concentrations, and varying types of tissues may also account for differences in data. Future research should assess ways to speed up the rate of diffusion without causing dye leakage.

NeuroVue dyes are lipophilic dyes similar to carbocyanines. Compared to these carbocyanines, however, Fritzsche *et al* (2005) suggests in a direct comparison study that NeuroVue Red is “superior to DiI with respect to visibility over longer diffusion distances”.<sup>7</sup> The NeuroVue Red and Maroon double labeling is purportedly better due to the fact that they both have similar tracing distances/speeds, whereas double labeling with DiI and DiA “gets complicated because of different diffusion times for each”.<sup>7</sup> NeuroVue dyes have a reduced duration necessary for diffusion. These studies involve similar tissue preparation methods to carbocyanines. For fixation and incubation solution, researchers most commonly used four percent PFA and 0.1 M PB. These dyes also need to be exposed to RT-37 °C to facilitate dye diffusion. We suggest more comparison studies by multiple independent labs to determine if NeuroVue dyes are superior to DiI.

The strengths of this review are that it is comprehensive in regards to a variety of variables that may impact the quality of axon tracing. A limitation of this review is the lack of recent published articles concerning axon tracing *ex vivo*. The lack of recently published research on new tracers may be due to the emergence of new imaging techniques such as dMRI tractography; however, this technique relies on the availability of specialized equipment and experimental software. With that said, we chose to focus our review on the more widely available histological methods of axon tracing. Additionally, due to limitations of systematic review methodology, perhaps the search terms missed potentially applicable studies as well as recently published studies. However, because of the use of ancestral searching, we are confident that we have identified the majority of the important studies.

## Conclusion

We conclude multiple methods are feasible for *ex vivo* axon tracing and the best histological method is dependent on the goals of the researcher. To each tracer and application method,

there are advantages and disadvantages in regards to cost, time, distance, specificity of tracing, single-cell labeling, ability to perform multiple labeling, and ease of use. Thus, what may be good for one purpose may not be good for another. According to the articles that were selected, DiI was reported to perform well in many aspects such as tracing length, ease of use, resolution, and price, as it is fairly inexpensive to use. A major disadvantage of DiI is that diffusion is slow, the order of weeks to months, which delays analysis. With the emergence of NeuroVue Dyes, some comparison studies have suggested superiority over the carbocyanine dyes; however, the majority of researchers still use DiI, which suggests more evaluations should be done to confirm superiority. Moreover, we suggest multiple independent labs conduct more comparison studies in order to definitively identify if this method yields better axon traced tissues.

## Acknowledgments

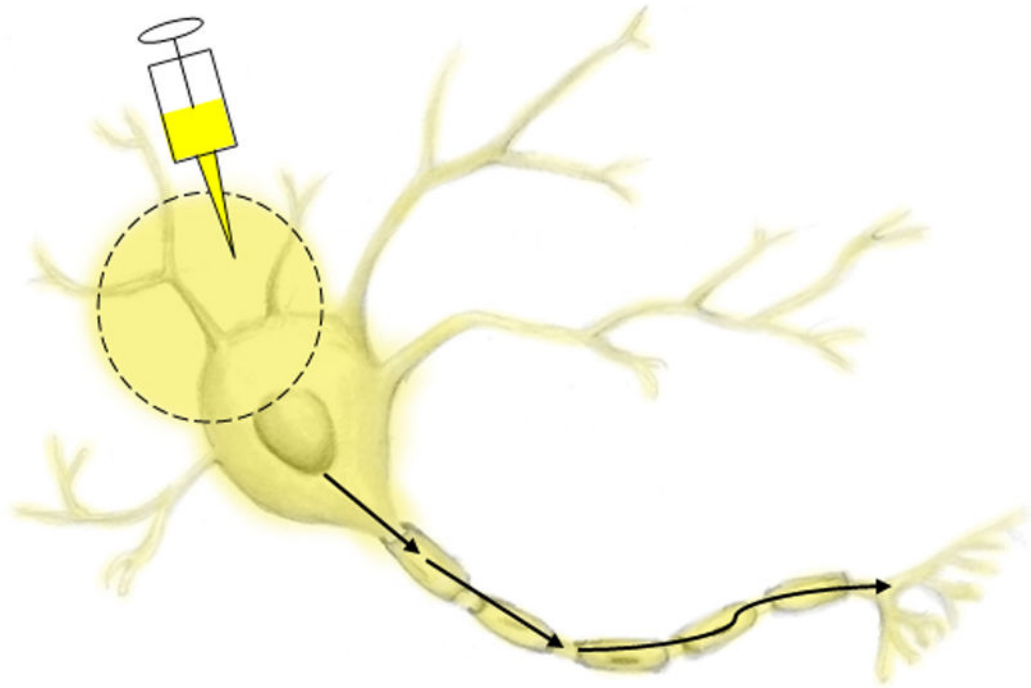
Supported by NIH grant 1K08NS079622

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# Anterograde

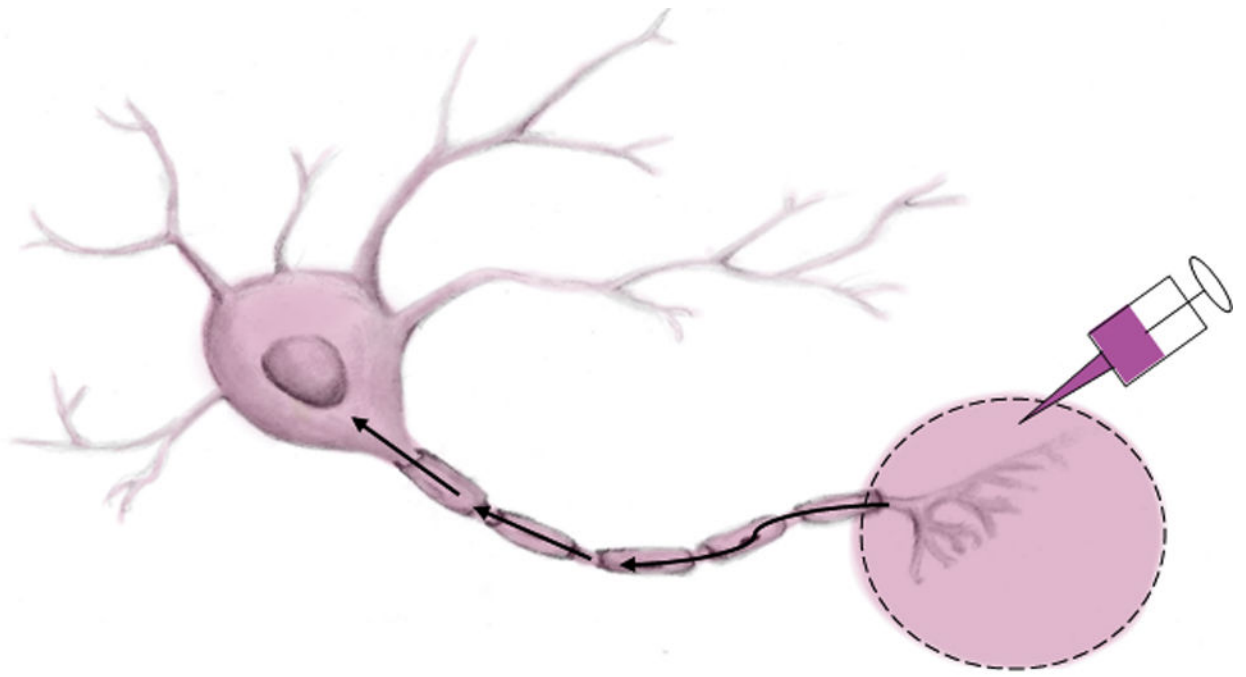
Figure 1.

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# Retrograde

Figure 2.

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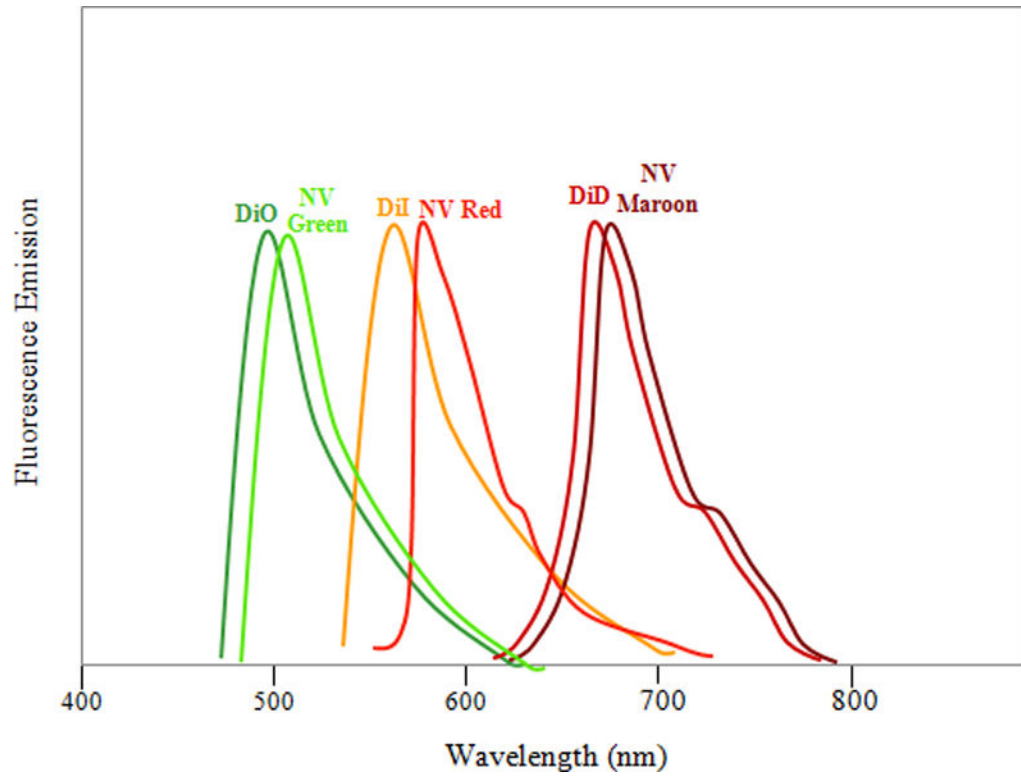


Figure 3.

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**ex vivo Axon Tracing Studies****Table 1.1**

This table summarizes the studies obtained from our search. Aspects such as tracer used, fixative, location, temperature, incubation solution, incubation duration, sectioning method, distance, and microscope used were all assessed from the studies in order to assist in comparing them.

Author/year	Tracer	Animal	Fixative	Location of Tracer/Tools	Incubation Temperature	Incubation Solution	Incubation Duration	Sectioning Method	Length Traced	Visualization
Anclair et al. 1993	DiI and DiA Crystals	13/14 day albino rat embryos	4% PFA	5–10 Crystals placed at C3 level of spinal cord to study brain stem. Method of insertion not specified.	37°C	4% PFA	2–4 months	Cryostat	Unclear	Epifluorescence Microscope
Baier et al. 1996	DiI and DiO	Zebrafish larvae	4% PFA	DiI and DiO injected using an apparatus to visualize retinotectal projection. DiI injected into temporal-ventral region and DiO injected into nasal-dorsal region	Room temperature	Unclear	Overnight	Unclear	Unclear	Fluorescence Microscope
Buhl 1989	Lucifer Yellow	Cats	4% PFA	Visual cortex	4°C	Unclear	Unclear	Vibratome	Unclear	Electron Microscope
Ceranik et al. 1999	DiI	Pre-natal and post-natal rats	4% PFA	Crystals placed on entorhinal cortex	Room temperature	4% PFA	3–6 weeks	Coronal sections 80–100 µm with vibratome	Unclear	Epifluorescence microscope/Confocal Laser Scanning Microscope
Chen et al. 2006	DiI and DiO	Adult Female Sprague Dawley Rats	4% PFA	Developed own method for insertion using 2mm thick and 4 mm diameter disc with 10 pins in it and placed these discs on either side of the spinal cord section and coated with wax to prevent leakage of dye	37°C and Room Temperature	1% and 4% PFA	2–24 weeks	Longitudinal vibratome sections	Distance varied depending on conditions. 37°C traveled up to 8.89±.23 mm over 24 wks And DiO traveled up to 1.67±0.11 mm in 4 weeks	Laser Scanning Confocal microscope
Collinge et al. 1991	DiI	Hamster	4% PFA	Cochlea. Placed crystals with micropipette.	40°C	Unclear	18–42 days	Transversely cut sections either 75 or 100 µm	Unclear	Epifluorescence
Eide et al. 1997	DiI	Chicken Embryo	4% PFA	Dorsal Root Ganglion	37°C	4% PFA	4–400 days	Vibratome	Unclear	Fluorescence Microscope
Friedland 1996	Fast DiI	Rat	4% PFA 0.1 M PB	Upper Respiratory Tract	37°C	4% PFA and 0.1 M PB	1 week	Vibratome	5–10mm	Epifluorescence
Fritsch 1990	DiI	Chicks	Unclear	Optic Nerve	21–25°C	2.5% formalin in 0.1M PB	4–28 weeks	Unclear	3 mm	Epifluorescence
Fritsch et al. 2005	DiI, DiA, DiO, DiD, NeuroVue Maroon, NeuroVue Red, NeuroVue Green	Mice	4% PFA	Auditory Cortex, Inferior Colliculus, Sacculle, Posterior Crista, Cerebellum, Brain Stem. NV has dye loaded filters	37°C	4%	24–96 hrs	Vibratome	Unclear	Epifluorescence
Haber. 1988	Horseradish peroxidase	Human	Unclear	Basal ganglia. Injected with a glass pipette	37°C and 4°C	Low calcium phosphate buffer or 10% glucose.	24 hrs	Microtome and vibratome	10mm from center of injection site	Unclear

Author/year	Tracer	Animal	Fixative	Location of Tracer/Tools	Incubation Temperature	Incubation Solution	Incubation Duration	Sectioning Method	Length Traced	Visualization
Hofmann and Bleckmann 1999	DII	Goldfish	4% PFA	Optic Nerve	6, 20, 30, 35, 40°C	10% fetal bovine serum and 10% penicillin streptomycin	1 week to 2 months	Vibratome	Unclear	Fluorescence
Jensen-Smith	NeuroVue Emerald, NeuroVue Jade, NeuroVue Red, NeuroVue Maroon	Mice embryos	Neutral-buffered PF (NBPF)	Spinal Cord Nylon filter	37–60°C	4% PFA, or 1–4% glutaraldehyde with 1% CaCl <sub>2</sub> and 3% potassium dichromate, or 1–4% glutaraldehyde in PB with 0.1% EDTA	24, 48, and 96 hours	Vibratome	Unclear	Confocal
Kayayama et al. 1987	HRP	Cats/goldfish	2.5% depolymerized PF and 1.5% glutaraldehyde	Central Nervous System. Inserted with a glass micropipette	4°C	0.1 M PB for the goldfish sections	1–2 weeks	Vibratome	1.5 mm	Electron microscope
Kikkawa 2007	DII, DiO, DiD, NV Red, and Golgi-Cox	Rat/mouse/guinea pig	Unclear	Nylon filters for NV Red. Looked at cochlea	Unclear	Unclear	NV Red 7–10 days. Golgi-Cox 3 weeks. Carbocyanine dye 4 weeks	Vibratome	Unclear	Confocal or light or Fluorescence
Lukas 1998	DII, DiO, DiA	Human cadavers and guinea pigs	4% PFA	Placed crystal with glass micropipette	37°C or 40°C	4% PFA	3 weeks to 1 year	Vibratome	≈29.2 mm in guinea pigs and 28.5 mm in human fixed tissue for DiI	Epifluorescence
Linke 1995	DII	Rat	4% PFA	Hippocamposeptal Projection	Unclear	4% PFA	4–12 weeks	Vibratome	Unclear	Fluorescent
Magoul 1994	DII	Rat	4% PFA	Arcuate nucleus	37°C	4% PFA	5 months	Vibratome and microtome	Unclear	Epifluorescence
Makarenko 2005	DII	Rat	4% PFA in 0.1M sodium phosphate buffer	Pituitary intermediate lobe	20°C	4% PFA	6–24 Months	Vibratome	Unclear	Fluorescent
Mastick 1996	DII and DiO	Mouse	4% PFA 0.1M PB	Forebrain and Midbrain	37°C	4% PFA 0.1M PB	4–24 hour	Unclear	Unclear	Dissecting Microscope
Mufson 1990	DII	Human	4% PFA	Hippocampal complex	20°C	4% PFA	6 months	Vibratome	8mm	Epifluorescence
Murphy et al. 2007	DII oil and crystal form	Mice	4% PFA	Vagal innervations	Unclear	4% PFA	4–7 weeks	Unclear	2–3.5mm	Fluorescent
Richards et al. 1997	DII dissolved in DMF	Rats	2% PFA and post fixed with 10% buffered formalin	Neocortex	RT	Unclear	>3 weeks	Vibratome	Unclear	Fluorescence
Soukup et al. 2009	NeuroVue Dyes	Mice	4% PFA	Inner ear	–	–	–	–	–	Confocal

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Author/year	Tracer	Animal	Fixative	Location of Tracer/Tools	Incubation Temperature	Incubation Solution	Incubation Duration	Sectioning Method	Length Traced	Visualization
Swift et al 2004	DII, DIO, DIA, DIR crystals dissolved in ethanol	Human cadavers	10% formalin	Peripheral nerve tissue	RT	Unclear	Up to 48 hours	Vibratome	12 hrs-15.4mm 24 hrs-29mm 48 hrs-53.7	Unclear
Zec 2001	DI	Human	4% PFA in 0.1 M PB with .05% Sodium Azide and 4% sucrose	Nucleus paragigantocellularis lateralis	20°C	Same as fixative	8.5-15.5 months	Unclear	Unclear	Fluorescent

Table 2

## Author's Conclusions for Comparison Studies

Author/Year	Comparison	Summarized Author's Conclusions
Haber 1998	Performed a tracing study with Wheat Germ Agglutinin Horseradish Peroxidase. Two incubation solutions and temperatures were used. The tissue was incubated in a culture medium containing 10% glucose, 10% fetal bovine serum, and 10% penicillin streptomycin or a calcium-low phosphate buffer at either 4°C or 37°C	<ul style="list-style-type: none"> <li>Not all cells/axons in a given area possessed labeling</li> <li>Axon and cell body labeling was enhanced under the 4°C conditions</li> <li>Under both the 37°C and 4°C conditions, the calcium-low phosphate buffer had better distance and staining than the culture medium</li> <li>The lower temperature enhanced the number of fibers seen and the distance traced.</li> </ul>
Kikkawa et al. 2007	This study compared three techniques to visualize the cochlear nerve fibers, namely DiI, the modified Golgi-Cox method and NeuroVue dyes.	<ul style="list-style-type: none"> <li>For the carbocyanine dyes, DiI, DiO and DiD were used. DiI was suggested to have the best staining among the carbocyanines</li> <li>The problem with the modified Golgi-Cox method was that it does not stain afferent fibers</li> <li>For all the different visualization methods tested, NeuroVue gave the best spatial resolution and fastest staining.</li> </ul>
Chen et al. 2005	Comparison study between DiI/DiO for the tracer, 1%/4% PFA for the preferred concentration, and RT vs. 37°C for the optimal temperature.	<ul style="list-style-type: none"> <li>DiI diffused substantially longer distances than DiO.</li> <li>Both dyes migrated farther distances at 37°C. Therefore, higher temperatures facilitate diffusion</li> <li>There was no difference between the tissue tracings incubated in 4% vs 1% PFA. However, 4% PFA was still required for the fixation and post fixation procedure.</li> </ul>
Lukas et al 1998	This study sought to optimize the axon tracing procedure in human and guinea pig tissue.	<ul style="list-style-type: none"> <li>Among DiI, DiA and DiO, DiI appeared to be the best tracer.</li> <li>The tissue was incubated at 37°C and 40°C for 3wks-1 year. After 15 weeks, no advancement was seen in the distance traced. Thus, the optimal tracing time was suggested to be 12–15 weeks. It was also suggested there was no difference between incubation at 37°C and 40°C.</li> <li>Since both central nervous system and peripheral nervous system tissue was traced in both guinea pig tissue and human tissue, no difference in tracing quality was seen in the two tissue types. However, a slight difference in distance was observed between the human and guinea pig tissue.</li> <li>Because carbocyanines are compatible with immunohistochemistry techniques, it was suggested Tween 20 was an optimal detergent than Triton X. It was also suggested Triton X may facilitate fading.</li> <li>In regards to sectioning, it was suggested cryostat sections may be less stable than vibratome sections.</li> <li>There was no observable difference between 2% PFA and 4% PFA for the incubation solution.</li> </ul>
Murphy and Fox 2007	This study compared DiI crystals to oil 0.1%, EDTA vs no EDTA, and PBS mounting medium vs glycerol and n-propyl gallate	<ul style="list-style-type: none"> <li>When comparing the DiI, they suggested crystals give a better quality and rate of diffusion</li> <li>The researchers suggest long incubation periods promote DiI leakage out of the nerve fiber.</li> <li>It is believed that calcium in the tissue promotes diffusion out of the neuron and promotes unspecific labeling. Therefore, in order to combat this, 0.1% EDTA is suggested to reduce this unspecific labeling.</li> <li>Lastly, PBS is suggested to be a superior mounting solution to glycerol with n-propyl gallate. Glycerol and n-propyl gallate reduced clarity and</li> </ul>

Author/Year	Comparison	Summarized Author's Conclusions
		<p>promoted leakage of the dye which ultimately results in a loss of specific labeling.</p>
<p>Hofmann et al 1999</p>	<p>The researchers aimed to improve the staining and limit transneuronal labeling. They investigated the effect of increased temperatures and calcium.</p>	<ul style="list-style-type: none"> <li>• The presence of calcium facilitates dye leakage out of the membranes; therefore, the addition of 0.1% EDTA to the 4% PFA eradicated transneuronal labeling.</li> <li>• In regards to temperature, 6°C, room temperature, 30°C, 35°C and 40°C, the researchers found that as the temperatures get higher, the staining becomes progressively more diffuse. It is suggested that increased temperatures accelerate dye diffusion out of the neuron.</li> </ul>
<p>Fritsch et al 2005</p>	<p>Compared NeuroVue Red, Green, and Maroon with DiI, DiO, DiA and DiD.</p>	<ul style="list-style-type: none"> <li>• DiO and DiA have slow diffusion in fixed tissue and are not as applicable as a long range tracer.</li> <li>• NV dyes were consistently seen to trace longer distances and have the ability to be imaged at lower concentrations than existing dyes. They also diffuse faster than the carbocyanines.</li> <li>• The red emitting and near infrared emitting dyes (NV Red, DiI, DiD, NV Maroon) were consistently seen to travel longer distances than the green emitting dyes.</li> <li>• NV Maroon appeared to be the easiest to detect over long distances</li> </ul>

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