Journal of Neuroscience Methods 141 (2005) 155–163

JOURNAL OF NEUROSCIENCE METHODS

www.elsevier.com/locate/jneumeth

Applied electric fields accelerate the diffusion rate and increase the diffusion distance of DiI in fixed tissue

Matthew J. Swift[a], Patrick E. Crago[a], Warren M. Grill[b,*]

[a] *Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106-7207, USA*
[b] *Department of Biomedical Engineering, Duke University, Box 90281, Durham, NC 27708-0281, USA*

Received 24 March 2004; received in revised form 7 June 2004; accepted 11 June 2004

Abstract

Lipophilic carbocyanine dyes are effective neuronal tracers in fixed tissue. However, their application has been limited by the slow diffusion, short tracing distances, and long durations of incubation in fixed tissue. We used applied dc electric fields, that exerted forces on the cationic dyes, to increase the diffusion velocity and maximal tracing distances of DiI and its analogs. Maximum diffusion distances of DiI in fixed human peripheral nerve were approximately 4 times longer then the previous reported maximum, and diffusion velocities was approximately 100 times faster in samples exposed to the electric field than in control samples. This method enabled retrograde tracing from a distal nerve branch into a proximal nerve trunk, and did not result in lateral transaxonal diffusion. Field enhanced diffusion will expand the range of uses of lipophilic dyes in fixed tissues and enable topographic mapping of peripheral nerve fascicles in post-mortem tissue.

Keywords: Neuronal tracing; Lipophilic dyes; Peripheral nerve; Neuroanatomy

1. Introduction

Lipophilic carbocyanine dyes, in particular DiI (1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate), are established as neuronal tracers in living tissue (Honig and Hume, 1986; Honig et al., 1989). Further, DiI is effective as both an anterograde (Godement et al., 1987) and retrograde (Vanselow et al., 1989) tracer in aldehyde fixed tissue. The chemical structure and highly lipophilic nature of these carbocyanine dyes allow them to diffuse within the plasma membrane of intact cells (Sims et al., 1974; Honig and Hume, 1986). However, in fixed tissue, the diffusion rates and maximum diffusion distances are quite limited. We developed and tested a method to accelerate the rate and increase the distance of DiI diffusion using applied dc electric fields.

DiI and the analogous dyes DiO (3,3′-dioctadecyloxacarbocyanine perchlorate), DiA (4-(4-(dihexadecylamino) styrl)-*N*-methylpyridinium iodide), and DiR (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide) are potential tools to document the fascicular anatomy of human peripheral nerves. The nature of the fascicular organization of human peripheral nerves has been a subject of debate (Stewart, 2003). Descriptions of intermingling of functional groups of axons within the human radial, median, and ulnar nerves as they were followed proximally (Sunderland, 1945) are in conflict with more recent studies showing that groups of sensory fibers remain clustered in the proximal human median nerve (Hallin, 1990) and that digital nerve branches maintain grouping throughout the course of the median nerve from the wrist to the shoulder in macaca mullata (Brushart, 1991).

The carbocyanine dyes meet many of the criteria necessary to perform studies of the fascicular organization in fixed human tissue. They diffuse retrogradely, work in fixed tissue, and have relatively stable fluorescence lives with reports of DiI stained neurons fluorescing 2 years after labeling (Elberger and Honig, 1990; Vidal-Sanz et al., 1988). The factor that limits the use of these dyes is the short tracing distances achieved in fixed tissue due to the increased

* Corresponding author. Tel.: +1-919-660-5131; fax: +1-919-684-4488.
E-mail address: wmgrill@duke.edu (W.M. Grill).

0165-0270/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jneumeth.2004.06.011

cross-linking of proteins during the fixation process (Sparks et al., 2000). The longest reported tracing distance for DiI in fixed tissue is 28.9 ± 2.2 mm after an incubation time of 12–15 weeks at 37 °C (Lukas et al., 1998). The same study revealed maximum tracing distances between 15 and 20 mm for DiA and DiO over the same incubation period. Lukas et al. detailed attempts to enhance diffusion via longer incubation times, increased incubation temperatures, and re-application of dye at the initial loading site, but none of these methods increased the maximum tracing distance.

We hypothesized that applying dc electric fields following dye application would increase diffusion rates and the maximum tracing distance of carbocyanine dyes in fixed tissue. The structures of DiI, DiO, DiA, and DiR are cationic and thus these molecules experience a force when placed in an electric field. That DiI will move due to a voltage gradient is supported by its application in studies of slow changes in cellular membrane potential (Sims et al., 1974; Grinvald et al., 1987; Zecevic and Antic, 1998) and the translocation of DiI molecules from one monolayer of a phospholipid bilayer membrane to the other in the presence of an applied electric field (Melikyan et al., 1996).

This report describes a method using applied dc electric fields to enhance the diffusion rate and maximum tracing distances of DiI, DiA, DiO, and DiR in fixed human peripheral nerve tissue. Substantial increases in tracing velocity and maximum tracing distances were observed for all of the molecules, and this method promises to increase the utility of these dyes as neuronal tracers in fixed tissue.

2. Materials and methods

2.1. Human tissue

Peripheral nerve samples were obtained from six fixed (10% formalin solution) cadavers (two male, four female) with an average age of 84 years (71–99). Median and ulnar nerve samples (diameters of 3–4 mm) were used, except when tracing from a branch into a main nerve was conducted, then cutaneous nerves (diameters of 0.5–3 mm) from the upper arm were used.

2.2. Tracers and their application

DiI, DiO, DiA, and DiR (Molecular Probes, Eugene, OR) crystals were dissolved in ethanol (1 mg/ml). Initial trials were also performed using the paste forms of DiI and DiO but no evidence of diffusion enhancement was observed, so the paste form was not pursued. A triangular notch, slightly deeper than the radius of the sample, was made into the nerve sample and 5–10 μl of the appropriate tracer solution was applied in this notch using a glass micropipette (Fig. 1). The solution was applied so that the majority of the tracer was on the side of the intended direction of diffusion, but application did not discriminate between the endoneurium and epineurium.

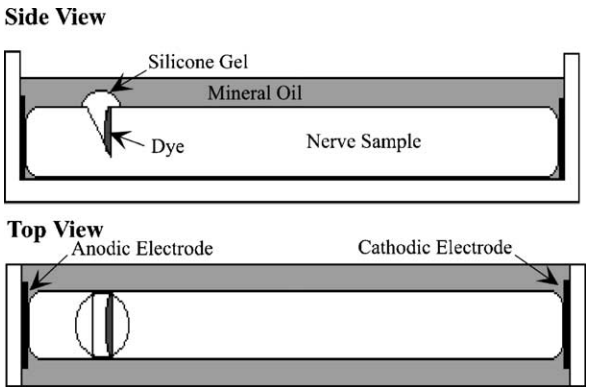

Fig. 1. Apparatus to apply longitudinal dc electric fields to samples of human peripheral nerves. Electric fields (10–40 V/cm) were applied between the anodic and cathodic platinum plate electrodes for 12–48 h, and the diffusion distance of the lipophilic dye, DiI, from the initial loading site was determined by serial transverse sectioning.

2.3. Field application

Platinum electrodes were used to apply a dc electric field across the nerve samples. Initially, 0.25 mm diameter wire electrodes were wrapped at both ends of the nerve with the electrode positioned closer to the initial dye application site serving as the anode. Although this was effective at generating a field within the nerve, maintaining a tight connection around the nerve without damaging the tissue and obtaining a precise distance between the two electrodes proved difficult. These two issues were successfully addressed by switching to parallel plate electrodes. Rectangular platinum plates 0.1 mm thick, 20 mm high, and 10 mm in width were positioned at both ends of the nerve sample which lay within a channel formed from plexiglass (Fig. 1).

Voltage was applied across the platinum electrodes using a high voltage power supply (EC-400, E-C Apparatus Corporation, St. Petersburg, FL). The field strength was set and monitored using two tungsten microelectrodes inserted into the nerve tissue 0.5 cm apart along the length of the nerve. The differential voltage was used to calculate the field strength within the nerve. During early trials, field strengths less then 5 V/cm were used, and this resulted in no clear effects on the diffusion velocity of DiI when applied for periods as long as 72 h. Subsequently, larger field strengths (as high as 70 V/cm) were tested. Field strengths larger then 45 V/cm frequently resulted in the nerve sample being forced away from the anodic electrode, and securing the nerve within the channel resulted in mechanical distortion of the tissue. However, his effect was never seen for field strengths at or below 40 V/cm, so 40 V/cm was chosen as the upper limit for further experiments.

Dehydration of the tissue during the tracing period was a concern throughout the experiments, in particular when thinner nerve samples were used. Initially, a silicone gel (Dow Corning* 7 Release Compound, Fisher Scientific) was used to coat the entire sample during the tracing period. How-