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# A double-transgenic mouse used to track migrating Schwann cells and regenerating axons following engraftment of injured nerves

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**Abstract**

We propose that double-transgenic *thy1-CFP(23)/S100-GFP* mice whose Schwann cells constitutively express green fluorescent protein (GFP) and axons express cyan fluorescent protein (CFP) can be used to serially evaluate the temporal relationship between nerve regeneration and Schwann cell migration through acellular nerve grafts. *Thy1-CFP(23)/S100-GFP* and *S100-GFP* mice received non-fluorescing cold preserved nerve allografts from immunologically disparate donors. *In vivo* fluorescent imaging of these grafts was then performed at multiple points. The transected sciatic nerve was reconstructed with a 1-cm nerve allograft harvested from a Balb-C mouse and acellularized via 7 weeks of cold preservation prior to transplantation. The presence of regenerated axons and migrating Schwann cells was confirmed with confocal and electron microscopy on fixed tissue. Schwann cells migrated into the acellular graft (163±15 intensity units) from both proximal and distal stumps, and bridged the whole graft within 10 days (388±107 intensity units in the central 4–6 mm segment). Nerve regeneration lagged behind Schwann cell migration with 5 or 6 axons imaged traversing the proximal 4 mm of the graft under confocal microcopy within 10 days, and up to 21 labeled axons crossing the distal coaptation site by 15 days. Corroborative electron and light microscopy 5 mm into the graft demonstrated relatively narrow diameter myelinated (431±31) and unmyelinated (64±9) axons by 28 but not 10 days. Live imaging of the double-transgenic *thy1-CFP (23)/S100-GFP* murine line enabled serial assessment of Schwann cell–axonal relationships in traumatic nerve injuries reconstructed with acellular nerve allografts.


*Keywords:* Transgenic mouse; Nerve allograft; Nerve regeneration; Schwann cell migration; Cold preservation; Live imaging

## Introduction

Static imaging, the mainstay for evaluating regenerating nerves after traumatic injury is limited by its inefficiency and inability to capture the dynamism of nerve regeneration and Schwann cell (SC) migration. Static imaging modalities including light or electron microscopy, immunohistochemistry, and retrograde labeling are resource intensive since they mandate the sacrifice of numerous experimental animals at multiple time points to characterize regeneration. Due to discrepant processing techniques, the application of one technique often precludes the use of the other techniques on the same specimen further increasing the number of experimental animals required to obtain quantitative data (Myckatyn et al., 2004). Further, none of these techniques, in isolation, sufficiently evaluates all of the parameters of regeneration such as rate, pathway sampling, and discrimination of motor versus sensory axons (Redett et al., 2005; Witzel et al., 2005). Characterization of regeneration following traumatic nerve injury in experimental models requires a technique capable of quantifying regenerating axons and migrating SCs over time in the same mouse to complement, if not replace, existing static imaging techniques.

Axons and SCs constitutively expressing a fluorescent reporter are key to an evolving methodology designed to

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track migratory and regenerative processes over time. Recent advances in molecular neurobiology provide us with transgenic mice expressing genes encoding fluorescent proteins under neuron-specific or SC-specific promoters. *Thy1-CFP(23)* mice, previously used to study axonal retraction in developing and maturing motor endplates express cyan fluorescent protein (CFP) in all motor, and many sensory axons (Feng et al., 2000; Walsh and Lichtman, 2003). *S100-GFP* mice are distinguished by SCs that are labeled with green fluorescent protein (GFP) under the control of transcriptional regulatory sequences of the human *S100B* gene (Zuo et al., 2004). A double-transgenic *thy1-CFP(23)/S100-GFP* line is referred to in an article reviewing the behavior of terminal SCs (Kang et al., 2003), but this work is not intended to evaluate regeneration through nerve grafts following traumatic injury. The relationships between SCs and axons during nerve regeneration are of particular interest to clinicians that reconstruct traumatic nerve injuries with grafts.

To advance the study of nerve regeneration and SC–axonal relationships, new models enabling longitudinal imaging of SCs and axons both within the nerve fiber and at the neuromuscular junction are needed. We previously report that mice expressing enhanced yellow fluorescent protein in their axons (*hThy1-EYFP*) can be serially imaged after traumatic nerve injury but do not provide methods for quantifying regeneration, or use confocal microscopy, and do not simultaneously image SC migration (Myckatyn et al., 2004). In this paper we compare the rates of SC migration and axonal regeneration into cold preserved, acellular allografts used to reconstruct a sciatic nerve transection injury in live *thy1-CFP(23)/S100-GFP* transgenic mice. We hypothesize that the *thy1-CFP(23)/S100-GFP* murine model can be used for serial, quantitative analysis of SC migration and axonal regeneration to study injured nerves repaired with acellularized nerve grafts.

## Materials and methods

### *Transgenic mice*

Heterozygous *thy1-CFP(23)* mice (Jackson, ME) were bred to homozygous *S100-GFP* mice ("Kosmos" line: gift from Dr. W. Thompson, Austin, TX, now commercially available through Jackson, ME). All SCs were diffusely labeled with GFP and the axons of 50% of the progeny were also labeled with CFP. We confirmed integration of the GFP transgene in the *S100-GFP* line visually by GFP expression in retinal glial cells and cutaneous Langerhans cells (Zuo et al., 2004). CFP transgene incorporation was confirmed in this heterozygous line using quantitative PCR on DNA extracts from mouse tail using the CFP primers provided by Jackson (forward, CTAGGCCACAGAATTGAAAGATCT-; reverse, GTAGGTGGAAATTCTAGCATCATCC). The mice were maintained in a central animal housing facility, and all described procedures were performed according to protocols approved by the Division of Comparative Medicine at the Washington University School of Medicine.

### *Cold preservation of nerve allografts*

SC migration and axonal regeneration were studied in interposed, 7-week cold preserved allografts, which are immunologically inert (Fox et al., 2005) and devoid of fluorescing cellular material (Myckatyn et al., 2004). Aliquots of the University of Wisconsin solution (15 ml; NPBI International BV, Emmer Compascuum, The Netherlands) with penicillin G (200,000 U/l), regular insulin (40 U/l), and dexamethasone (16 mg/l) were dispensed into sterile petri dishes. 1.5-cm Sciatic nerves from Balb/C mice were transferred into solution directly after harvest and maintained at 4 °C for 7 weeks to eliminate any viable antigenic cells (Fox et al., 2005).

### *Surgical procedure*

The eighteen mice used for this experiment included *thy1-CFP(23)/S100-GFP* ($n=4$) and *S-100 GFP* ($n=8$) transgenic mice which received nerve allografts and Balb/C mice ($n=6$) that donated allografts. Animals were anesthetized with a subcutaneous injection of ketamine (75 mg/kg) and medetomidine (100 mg/kg). A skin incision was made parallel and 2 mm posterior to the femur and the sciatic nerve exposed with microsurgical technique to include the sciatic notch proximally and its trifurcation to tibial, peroneal, and sural nerves distally. The sciatic nerve was transected and a 1.0-cm cold preserved nerve allograft from a Balb/C mouse interposed between the transected ends in a reversed orientation with 11–0 microsutures. Imaging was performed, muscle and skin closed in layers with 6–0 and 8–0 nylon sutures (Ethicon, NJ) and mice recovered with Antisedan (Novartis, Canada) on a warming pad.

### *Live imaging*

We serially imaged the nerve grafts at the time of surgery, and 5, 10, 15, and 28 days later using a fluorescence-enabled dissecting microscope (Nikon SMZ 1500 with Applied Scientific Instrumentation Z-stage) (Myckatyn et al., 2004). To facilitate live imaging, mice were re-anesthetized and the grafted sciatic nerve re-exposed and imaged with the fluorescent dissecting microscope. Images were recorded with a CoolSNAP-ES cooled CCD camera (Photometrics, Tucson, AZ) utilizing MetaMorph version 6.2 (Universal Imaging Corporation, Downingtown, PA) image acquisition software. Specimens were evaluated under GFP (488 nm: SCs) and CFP (450 nm: axons) fluorescent cubes, and imaged with a monochromatic CCD and MetaMorph. Images were initially standardized in terms of magnification (40×), exposure time (200 ms), and orientation. The relationship between exposure time and image fluorescent intensity was also standardized to Evicomposite (Evident Technologies, Troy, NY), polymer resin embedded with quantum dots (EviDots) that emitted stable nanocrystalline fluorescence at a wavelength of 493 nm.

GFP and CFP fluorescent intensity along the length of the nerve graft was quantified using the "line scanning" technique described below. Following image acquisition, the wound was