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*Full Length Research Paper*

# Simultaneous visualization for coexpression of multiple neurotrophic factors in living Schwann cells

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**Schwann cells, as specialized glial cells found in the peripheral nervous system (PNS), produce a variety of neurotrophic factors (NTFs) and play a vital role in maintaining PNS functions. The combined biological effects of multiple NTFs are strongly associated with their coexpression characteristics in the physiological environment of living cells. In this study, the method for visualizing coexpression of multiple NTFs in living Schwann cells was investigated. We isolated Schwann cells from rat sciatic nerve and co-cultured them with dorsal root ganglion (DRG) neurons. The DRG neurons were removed, following which the Schwann cells were hybridized with fluorescence-labeled oligonucleotide probes for nerve growth factor (NGF), ciliary neurotrophic factor (CNTF) and glial cell line-derived neurotrophic factor (GDNF) introduced by electroporation and observed under confocal laser scanning (CLS) microscope. Our experimental results revealed that the following factors were crucial for visualizing the coexpression of multiple NTFs in living cells: (1) probe design and labeling, (2) probe specificity, (3) electroporation parameters for introducing probes into living cells and (4) hybridization signal detection. Our study may provide further insights into the synergistic effects of these factors on neurons.**

**Key words:** Coexpression, neurotrophic factor, Schwann cell, visualization.

## INTRODUCTION

Neurotrophic factors (NTFs) are secreted peptides that promote axonal regeneration and play important roles in maintaining survival of neurons. Most NTFs belong to one of the three major NTF families, namely, the neurotrophin, neuropoietic cytokine and glial cell line-derived neurotrophic factor families (Frostick et al., 1998). Schwann cells are specialized glial cells found in the peripheral nervous system (PNS) and they produce many types of NTFs such as NGF, CNTF and GDNF (Frostick et al., 1998; Watabe et al., 1995). NGF maintains the survival of embryonic DRG neurons and increases the activity of the sensory and sympathetic neurons (Frostick et al., 1998; Deckwerth and Johnson, 1993). CNTF promotes neurite growth from sensory and sympathetic neurons and supports the survival of motor neurons (Arakawa et al., 1990, Magal et al., 1991). GDNF functions in maintaining the survival of dopaminergic neurons in mammals and in preventing apoptosis and atrophy of motor and sensory neurons (Henderson et al., 1994; Lin et al., 1993; Matheson et al., 1997). Many studies have shown that NTFs belonging to different families provide both selective and cross protection to neurons (Terenghi, 1999). In most cases, various NTFs act on the same neuron to produce synergistic and complementary effects (Cao and Shoichet, 2003; McCallister et al., 2001; Vejsada et al., 1998; Zurn et al., 1996). Moreover, some NTFs influence the effects of other NTFs by regulating the expression of these NTF receptors (Hagg et al., 1992). Further, interactions among various NTFs may also occur (Zhong

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**Abbreviations: PNS,** Peripheral nervous system; **NTFs,** neurotrophic factors; **DRG,** dorsal root ganglion; **NGF,** nerve growth factor; **CNTF,** ciliary neurotrophic factor; **GDNF,** glial cell line-derived neurotrophic factor; **CLS,** confocal laser scanning; **RT-PCR,** reverse transcriptase- polymerase chain reaction; **DMEM,** dulbecco's modified eagle's medium; **FBS,** fetal bovine serum; **GGF,** glial growth factor; **FISH,** fluorescence *in situ* hybridization; **PBS,** phosphate-buffered saline; **BSA,** bovine serum albumin.

et al., 1994). These additive or synergistic effects may be strongly associated with the levels at which multiple NTFs are coexpressed in the physiological environment of living cells (Deister and Schmidt, 2006).

Expression of a single NTF has been reported in numerous items of literature (Frostick et al., 1998; Saika et al., 1991; Friedman et al., 1992; Lee et al., 1995; Hoke et al., 2000; Sebert and Shooter, 1993). However, little is known about the characteristics of multiple NTF coexpression in the physiological environment of living Schwann cells. Previous *in vivo* or *in vitro* studies on the expression of NTFs in Schwann cells have detected their expressions by using methods such as RT-PCR, Western blotting, northern blotting, or immunocytochemistry (Lee et al., 1995; Hammarberg et al., 1996; Marcinkiewicz et al., 1999). However, these methods involve processes such as nucleic acid extraction or sample fixation that inevitably alter and may even destroy the inherent structural content of the samples (Krylov et al., 2000). Cavallo et al. (2002) performed fluorescence labeling for specific nuclear DNA sequences of neuroblastoma cells and demonstrated a certain degree of migration of fluorescent particles inside the cell nucleus in the fixed-cell state or during fluorescence in situ hybridization after fixation as compared to the living-cell state. Therefore, the detection of gene expression in the living-cell state may, to a certain extent, prevent the interference caused by sample processing.

Simultaneous visualization of multiple NTFs in living Schwann cells may provide further insights into the synergistic effects of these factors on neurons. We aimed at establishing a novel method for visualizing and detecting the coexpression of multiple NTFs in living Schwann cells. In this study, oligonucleotide probes for NGF, CNTF and GDNF were synthesized and labeled with fluorescent dyes of distinct spectra, introduced into living Schwann cells by electroporation, visualized under confocal laser scanning microscope system and analyzed by using the Leica Confocal software.

## MATERIALS AND METHODS

### Animals

Three-day-old Sprague-Dawley rats with 15 days pregnancy were provided by the Experimental Animal Center, Third Military Medical University, Chongqing, China. All animals were housed in a standard animal facility on a 12 h light/12 h dark cycle, with free access to food and water. All procedures were performed in accordance with the Animal Care Guidelines of Third Military Medical University, which conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study was approved by the ethics committee of our university.

### Schwann cells and DRG neuron cultures

Schwann cells and DRG neurons were isolated and purified using a previously described process with slight modifications (Einheber et al., 1997). Schwann cells were isolated from the sciatic nerve of rats on postnatal day 3 and maintained in standard media consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Hyclone), 10% fetal bovine serum (FBS; Hyclone), 100 u/ml penicillin and streptomycin. The purified Schwann cells were amplified in standard media supplemented with 5 ng/ml glial growth factor (GGF) and 4 μM Forskolin (Sigma). Further, approximately 200, 000 Schwann cells were seeded onto a polylysine-coated 12 mm coverslip and kept in standard media. DRG neurons were isolated from the dorsal root ganglion of rat embryos at 15 day gestation. The purified DRG neurons were plated onto a polylysine- and laminin-coated 12 mm coverslip at a density of approximately 5,00 - 10,000 neurons per coverslip and maintained in a neurobasal medium (Gibco) supplemented with 2% B27 (Gibco) and 50 ng/ml 2.5S NGF (Sigma).

The Schwann cells and DRG neurons on the coverslips were cocultured in 35 mm plates at a 2 mm distance interval from each other. The co-cultures were maintained in the neurobasal medium supplemented with 2.5% FBS, 2% B27 and 50 ng/ml 2.5S NGF for 6 days, following which the coverslip containing DRG neurons was removed.

### Design of oligonucleotide probes

Oligonucleotide probes complementary to mRNAs for rat NGF, CNTF and GDNF were designed using the Primer 5.0 software, on the basis of published nucleotide sequences. Further, the specificity of the probes was observed by using the National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) software (http://www.ncbi.nlm.nih. gov/BLAST). The probes were synthesized and labeled with Fam, Cy3 and Cy5 dyes, which were obtained from Shanghai Bioasia Corporation, in the following format: NGF, Fam-5′-AACAGGACTCACAGGAGCAAGC GGTCATCATCCCATCCCATCTTCCACAGG-3′; CNTF, Cy3-5′-GG CTGATGGGATGCCTGCCACAGTTGGAGATG-3′ and GDNF, Cy5-5′-CGGGACTCTAAGATGAAGTTATGGG ATGTCGTGGCTGTCTG CCTGGGT TGC-3′.

### Probe specificity studies by fluorescence *in situ* hybridization in fixed Schwann cells

For testing the specificity of the probes, fluorescence *in situ* hybridization (FISH) was performed in fixed Schwann cells. The Schwann cells were fixed with 4% paraformaldehyde, subsequently washed with phosphate-buffered saline (PBS) and finally a Multicolor FISH method previously described (Takada et al., 2004) was applied with the hybridization solution containing the three fluorescence-labeled probes for NGF, CNTF and GDNF at a concentration of 2.5 μmol/l. In control experiments, the Fam-, Cy3- and Cy5-labeled bovine serum albumin (BSA) 40 μg/ml was used to determine non-specific probe binding. The fluorescent signals produced in the Schwann cells were detected by using a Leica TCS SP2 confocal laser scanning (CLS) microscope (Leica Microsystems, Heidelberg, Germany). Samples were excited at 488, 543 and 633 nm and emitted light was filtered at 522, 563 and 662 nm for Fam, Cy3 and Cy5, respectively. Fam, Cy3 and Cy5 were assigned to green, red and blue, respectively, on the basis of the RGB color model.

### Transfer of NGF, CNTF and GDNF probes into living schwann cells by electroporation

For the introduction of NGF, CNTF and GDNF probes into living Schwann cells, electroporation was used. The Schwann cells were prepared for electroporation with the probes. 2.5 μmol/l of the fluorescence-labeled probe was used for NGF, CNTF and GDNF,

respectively, in a 4 mm gap cuvette at various electric field strengths (0.5, 0.75 and 1.0 kV/cm) and different pulse durations (10 and 20 ms) in order to identify the optimal electroporation parameters, with which the best electroporation efficiency could be obtained. Fluorescent images were captured by using the CLS microscope 24 h postelectroporation. The fluorescent cells were counted for measuring the electroporation efficiency. Further, staining with 0.4% Trypan Blue (Gibco) was used to calculate the percen-tage of viable cells by using an Olympus DP71 microscope equipped with a digital camera system (Tokyo, Japan). The cell survival rate (%) and electroporation efficiency (%) were calculated, respectively, as the ratio of the number of unstained cells, or the fluorescent cells to the total number of cells.

#### Evaluation of the specificity of probe binding in living Schwann cells

For testing the specificity of probe binding in living Schwann cells, the unlabelled probes for NGF, CNTF and GDNF were mixed, respectively, with its fluorescence-labeled probes to obtain a probe mixture for each factor. Identical final concentration of 2.5 μmol/l for both the unlabelled probes and fluorescence-labeled probes were made up in the mixture. The optimal electroporation parameters were applied for introducing the probe mixture or the fluorescence-labeled probes of each factor inside living Schwann cells. The specificity of the probe binding in living Schwann cells was evaluated by comparing the fluorescent intensity yielded by the probes in the mixture with those yielded by the fluorescence-labeled probes alone. The observation and acquisition of fluorescent images were performed with the CLS microscope and the Leica Confocal software was used for the measurement of fluorescent intensity. Data analysis was performed using a two-tailed unpaired Student's t-test with the SPSS program version 12.0 for Windows (SPSS Inc., Chicago, IL, USA). A P value < 0.05 was considered statistically significant.

#### Optical sectioning and 3D reconstruction by CLS microscope

The coexpression of NGF, CNTF and GDNF mRNAs was conducted by introducing the fluorescence- labeled probes for NGF, CNTF and GDNF at a final concentration of 2.5 μmol/l simultaneously with the optimal electroporation parameters. Confocal optical sectioning was performed by using the CLS microscope and images were taken with 1 μm step size. The transmitted light images of the Schwann cells and the emission signals excited at 488, 543 and 633 nm were obtained, respectively. The three fluorescent signals were superimposed to generate images of the coexpression of NGF, CNTF and GDNF mRNAs. Moreover, the fluorescent images of the coexpression were overlaid onto the transmitted light images to visualize the expression distribution in the Schwann cells. The three-dimensional (3D) images of GDNF mRNA expression were reconstructed from sequential optical sectional images. All procedures were performed by means of the Leica Confocal software.

## RESULTS

### Specificity of the probe

In order to verify the specificity of the probes, the fixed Schwann cells were subjected to FISH with the fluorescence-labeled probes for NGF, CNTF and GDNF simultaneously, with the fluorescence-labeled BSA being used as a control. The characteristic staining caused by Fam-, Cy3- and Cy5-labeled probes for NGF, CNTF and GDNF, respectively, were observed (Figure 1A). However, cells did not show fluorescent characteristic regions of specific staining when Fam-, Cy3- and Cy5-labeled BSA were used in control experiments (Figure 1B).

### Optimal electroporation parameters

Our experiments revealed that the electroporation efficiency was higher with electric field strength of 0.75 kV/cm and pulse duration of 20 ms at the probe concentrations of 2.5μmol/l, whereas the best cell survival rate did not occur under this condition. The cell survival rates after electroporation of the probes for NGF, CNTF and GDNF exhibited an identical characteristic and so did the electroporation efficiencies. Figure 2 illustrates the mean values of cell survival rates, as well as the electroporation efficiencies of three independent electroporation experiments of NGF, CNTF and GDNF probes, respectively, with various electric field strength and pulse duration.

### Specificity of probe binding in living Schwann cells

As shown in Figure 3, the values of the fluorescent intensity of hybridization signals in living Schwann cells yielded by the mixture of fluorescence-labeled probes and unlabelled probes for each of NGF, CNTF and GDNF at a concentration of 2.5 μmol/l decreased significantly than those yielded only by the fluorescence-labeled probes at the same concentration (P < 0.01 for NGF, CNTF and GDNF, respectively).

### Confocal images of NGF, CNTF and GDNF coexpression and 3D pattern

The coexpression of NGF, CNTF and GDNF mRNAs in different cell sections was examined by subjecting the Schwann cells to sequential optical sectioning with a CLS microscope. The fluorescent region was maximal in the center of the cells body and became small toward the periphery. The expression distributions of the three factors differed within the same section. Overlapping fluorescent staining was seen for all the three factors (white) as well as for two of the three factors (magenta for CNTF and GDNF mRNA, cyan for NGF and GDNF mRNA). Singular mRNA expression of a single factor was also observed in certain areas (Figure 4A). Overlay of bright-field and fluorescent images of NGF, CNTF and GDNF mRNAs coexpression displayed the expression distributions in the Schwann cells (Figure 4B). Further, the 3D images of GDNF mRNA expression (Figure 5A) reconstructed from sequential optical sections (Figure 5B) provided different viewing angles of the intact pattern

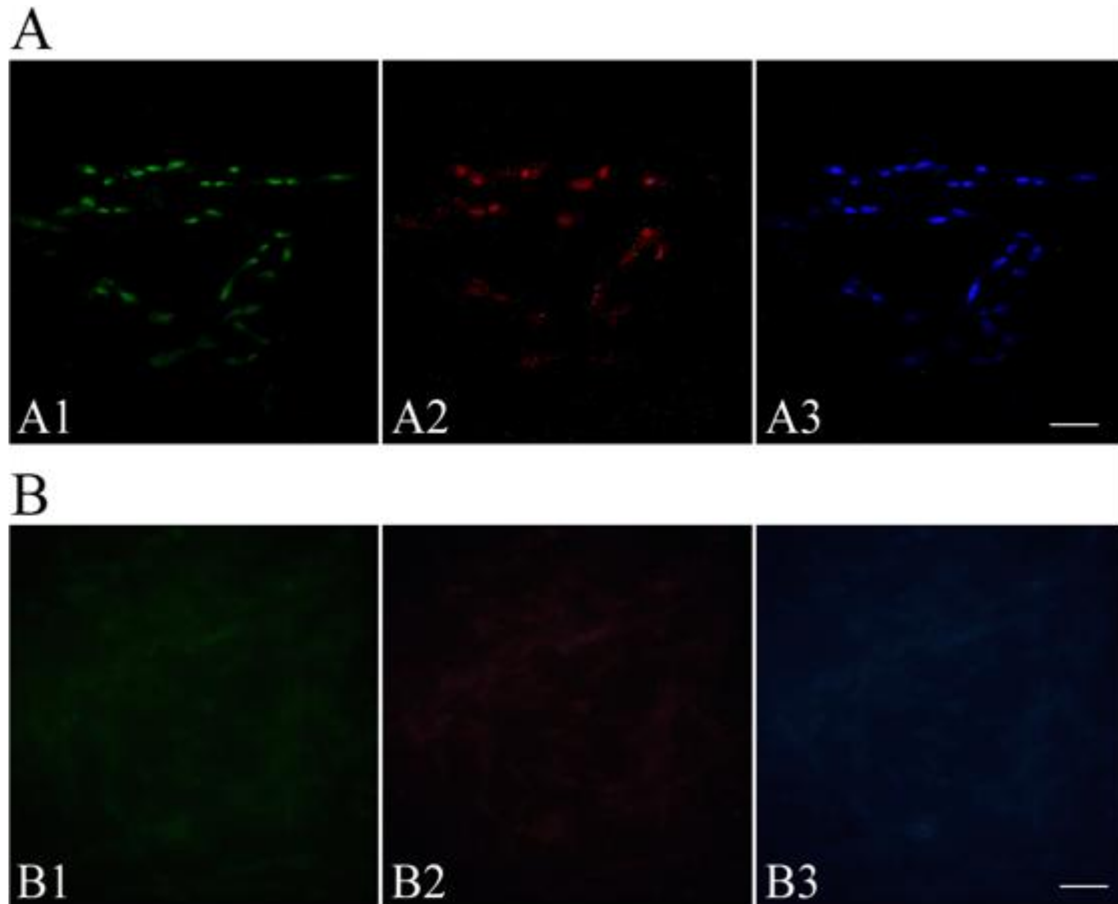


**Figure 1.** Hybridization of fluorescence-labeled probes for NGF, CNTF and GDNF, as well as their controls by using fluorescence-labeled BSA in fixed Schwann cells. (A) Hybridization of Fam-, Cy3- and Cy5-labeled probes for NGF (A1), CNTF(A2) and GDNF (A3). Characteristic staining caused by each probe was visible. (B)Hybridization of Fam-(B1), Cy3-(B2) and Cy5-labeled BSA (B3). Characteristic staining was not observed in this case. Scale bar: 200 μm.

of mRNA expression in a single Schwann cell. However, typical cytoplasmic mRNA expression was observed in this figure.

## DISCUSSION

Investigations are increasingly focusing on the synergistic effects of multiple NTFs during the repair of nerve injury (Cao and Shoichet, 2003; Deister and Schmidt, 2006; Logan et al., 2006; Ogilvie et al., 2000; Sharma, 2007). Analysis of the coexpression of multiple NTFs in living Schwann cells could provide further insights into the synergistic functions of these factors in neurons. In this study, we investigated a method for visualizing the co-expression of NGF, CNTF and GDNF mRNAs in living Schwann cells. Our results suggested that the following factors are crucial for visualizing the coexpression of multiple NTFs in living cells: (1) probe design and labeling, (2) probe specificity, (3) electroporation parameters for introducing probes into living cells and (4) hybridization signal detection.

### Probe design and labeling

Due to the instability of probes in living cells and the fact that nonhybridized probes cannot be washed away, the probe sequence and length should be carefully considered. We used oligonucleotide probes in this experiment since they had the specific characteristics required for molecular hybridization in living cells, such as short length, low sequence complexity and low molecular weight. These characteristics ensure hybridization with high speed and strong specificity, thus reducing probe instability in living cells to a certain degree. The mRNA expressions of multiple NTFs can be detected simultaneously by labeling the probes for these NTFs with various fluoresceins in distinct excitation and emission spectra, using the specific color-adjustment method.

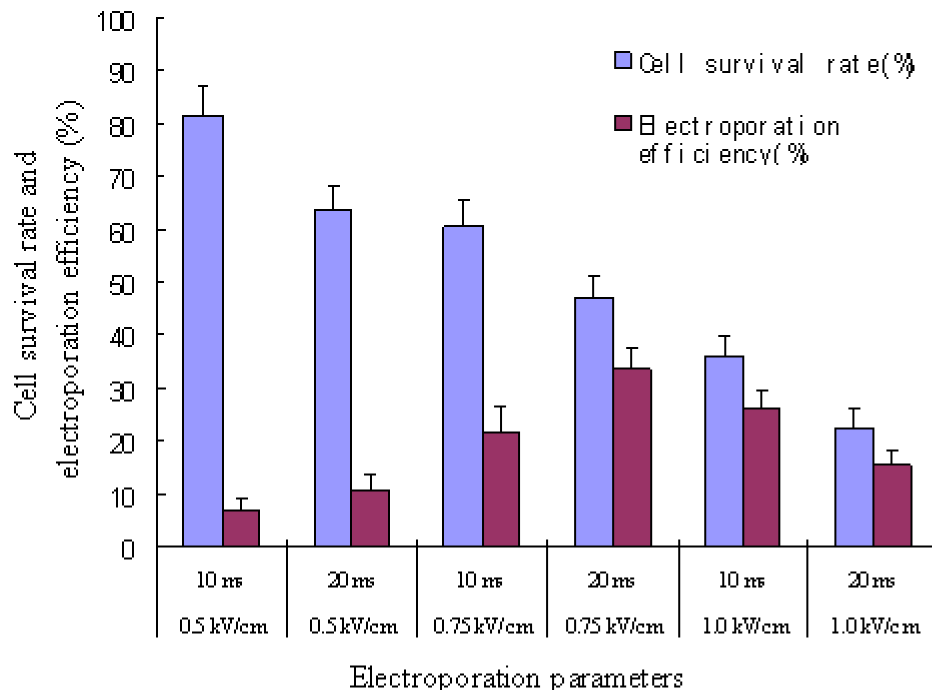


**Figure 2.** Cell survival rates (%) and electroporation efficiencies (%) after electroporation of the fluorescence-labeled probes for NGF, CNTF and GDNF at a concentration of 2.5 μmol/l. Each value in the graph represents means ± standard deviation of three independent electroporation experiments of NGF, CNTF and GDNF probe, respectively. The best electroporation efficiency was obtained with an electric field strength of 0.75 kV/cm and pulse duration of 20 ms.

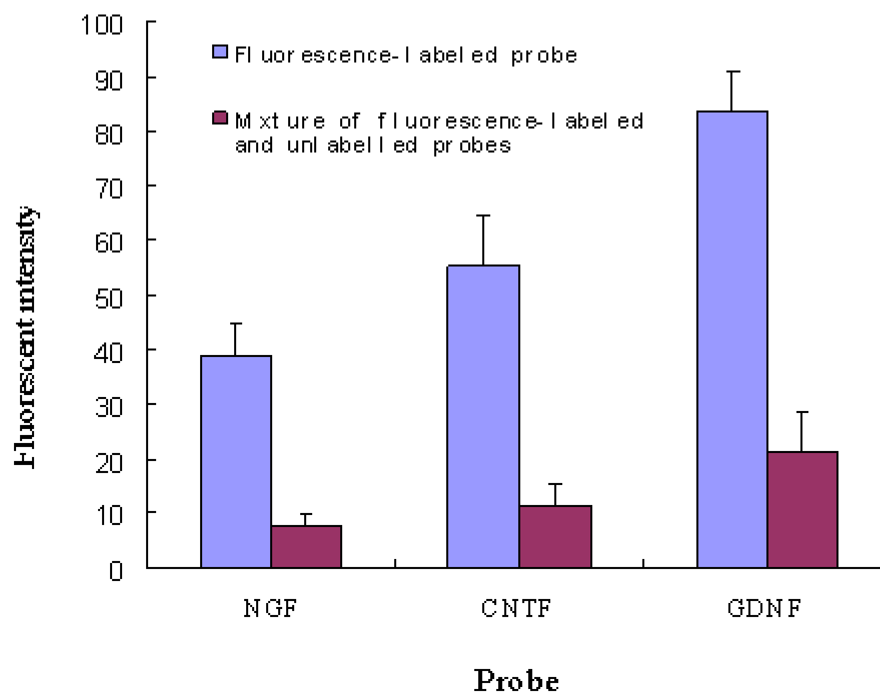


**Figure 3.** Fluorescent intensity of hybridization signals in living Schwann cells produced by the mixture of fluorescence-labeled and unlabelled probes decreased significantly compared with that produced only by the fluorescence-labeled probes for NGF($P < 0.01$), CNTF($P < 0.01$) and GDNF($P < 0.01$), respectively. Values are mean ± SE.

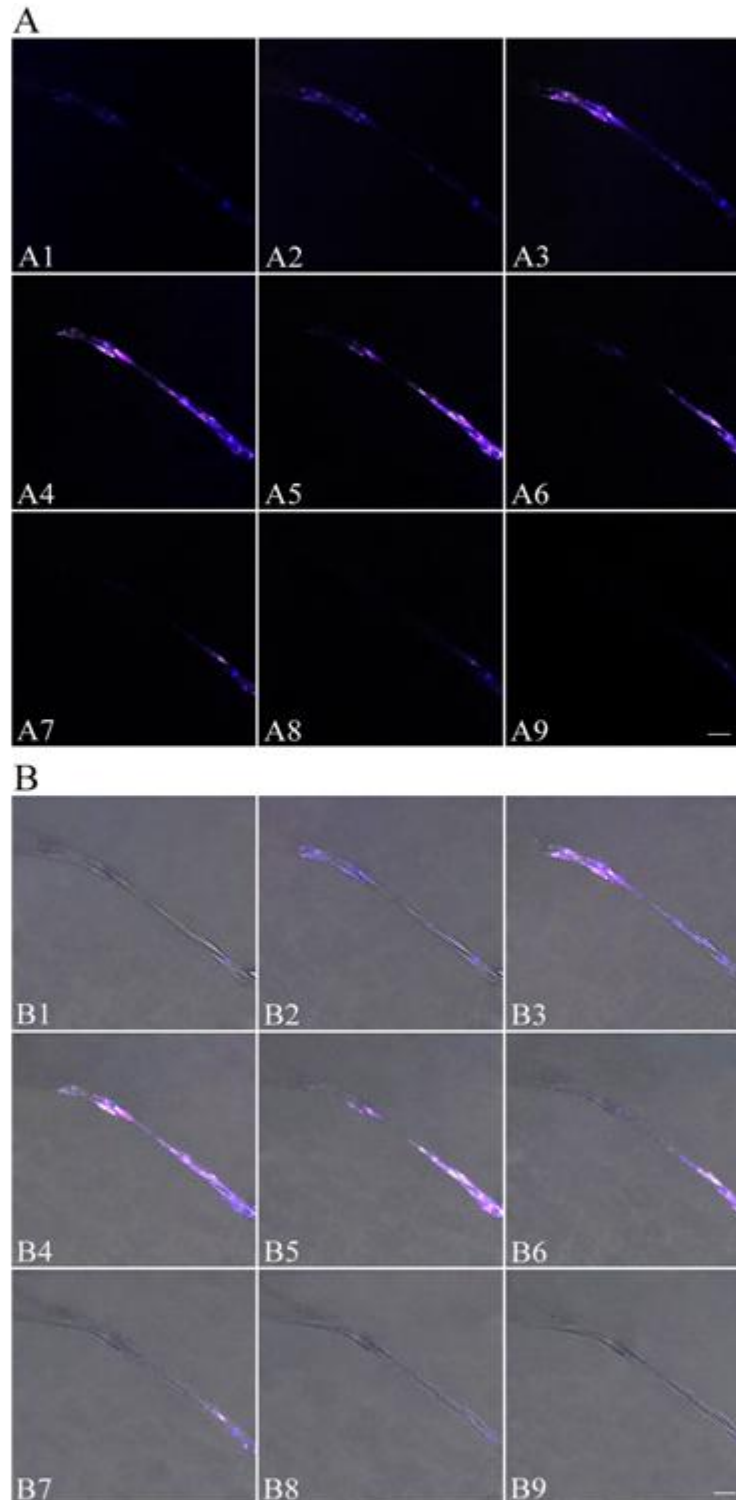


**Figure 4.** Confocal scans of the coexpression patterns of NGF, CNTF and GDNF mRNAs in living Schwann cells. (A) Sequential sectional images of the coexpression of NGF, CNTF and GDNF mRNAs. (B) Overlay of bright-field and fluorescent images in (A). The expression distributions of the three factors (green for NGF mRNA, red for CNTF mRNA, blue for GDNF mRNA) differed within the same section. Overlapping fluorescent staining was seen for all the three factors (white) as well as for two of the three factors (magenta for CNTF and GDNF mRNA, cyan for NGF and GDNF mRNA). Scale bar: 80 μm.

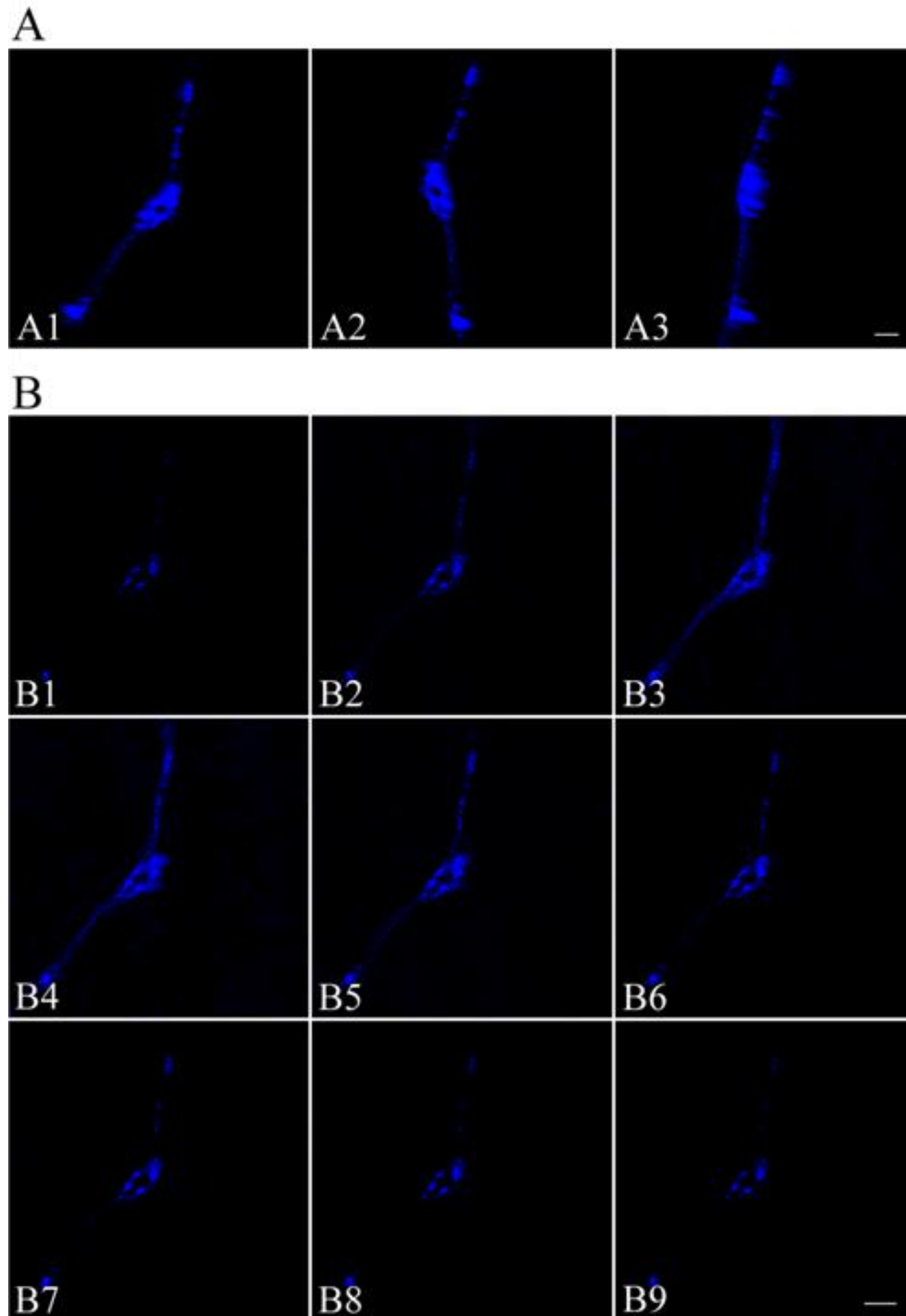


**Figure 5.** 3D reconstruction of GDNF mRNA expression in a single Schwann cell. (A) Different viewing angles of the reconstructed 3D images exhibited the intact patterns of GDNF mRNA expression in a single Schwann cell. Typical cytoplasmic mRNA expression was observed. (B) Sequential optical sections used for the image reconstruction in (A) taken by CLS microscope. Scale bar: 40 μm.

**Evaluation of probe specificity**

To ensure that the distribution patterns of multiple NTFs expression observed in living cells are not nonspecific hybridization to nontarget sequences or distribution patterns of nonhybridized probes, control experiments

should be performed both in living cells as well as in fixed cells (Dirks et al., 2003). Fluorescence- labeled BSA was used as a control of probes for NGF, CNTF and GDNF in the FISH experiments conducted in fixed Schwann cells. However, no fluorescent region of specific staining was observed in the presence of Fam-, Cy3- and Cy5- labeled BSA, whereas characteristic staining was seen when fluorescence-labeled probes for NGF, CNTF and GDNF were used. Therefore the specificity of probe was demonstrated. Furthermore, on account of the inability to wash out nonhybridized probes in living cells, hybridization in living cells with a mixture of unlabeled and fluorescence-labeled probes, as well as with fluorescence-labeled probes at the same concentration as that in the probe mixture were performed to determine whether the fluorescent signals arose from nonhybridized free diffusing probes. The results of our experiments revealed that the hybridization signals yielded by the probe mixture in living cells were significantly weaker than those yielded by the fluorescence-labeled probes. Based on the fact that specific hybridization patterns might become less intense or even may disappear due to competition between the unlabeled and fluorescence-labeled probes for binding target sequences, the fluorescent signals observed in living Schwann cells were proved to be specific hybridization patterns other than distribution patterns of nonhybridized fluorescent probes.

## Electroporation parameters for introducing probes into living cells

By increasing the cell permeability, probes of multiple NTFs can be simultaneously transferred into living Schwann cells by using electroporation (Golzio et al., 2004). The cell viability and electroporation efficiency of probes may be directly influenced by electroporation parameters such as electric field strength and pulse duration used. Probes may fail to enter the cells if the surface state of the cell membrane remains unaltered due to low voltage or short pulse duration. On the other hand normal hybridization between the transferred probes and their targets in the cells may also fail to occur in the event of denaturalization or death of the cells due to excessive heat accumulation resulting from the use of a high voltage or prolonged duration of electric pulse. Our experiment demonstrated that transfer of probes into living Schwann cells by electroporation require comparative lower electric field strength and longer pulse duration. In our experiments, we determined the suitable electroporation conditions for achieving the optimal electroporation efficiency by comparing the electroporation efficiencies obtained at different field strength and pulse duration, namely, 0.5 kV/cm (10 and 20 ms), 0.75 kV/cm (10 and 20 ms) and 1.0kV/cm (10 and 20 ms). The best electroporation efficiency was obtained with an electric field strength of 0.75 kV/cm and a pulse duration of 20ms. Under conditions of a given electroporation parameter, the probe concentration is a key factor influencing the signal-to-noise ratio of hybridization images. According to previous report that optimal signal-to-noise ratio of hybridization images in living cells may be obtained at a final probe concentration of not more than 5.0 μmol/l (Dirks et al., 2003), 2.5 μmol/l probes were applied and better signal-to-noise ratio was obtained in our experiments.

## Detection of the hybridization signals

Compared with conventional light microscopy, CLS microscope can overcome the limitation of overlapping of the sample structures and permit better observation by producing optical sectional images and converting *in situ* expression data into 3D patterns (Sugawara et al., 2005). Moreover, fluorescent images and transmitted light images may be obtained simultaneously, thus, the distribution patterns of fluorescence signals corresponding to the coexpression of NGF, CNTF and GDNF mRNAs in Schwann cells were visualized by overlay of these images.

In recent years, new techniques for visualization of gene expression in real time have been increasingly valued by scientists and studies have intensively focused on the visualization of gene expression in living cells (Rodriguez et al., 2006). Here, we demonstrated that the integrity and activity of cells can be maintained during the visualization of multiple NTFs coexpressions in living Schwann cells, without any interference caused by sample processing. We hope that this study leads to the development of a novel method for investigations on how Schwann cells and their NTFs promote the repair and regeneration of injured nerves.

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