Growth Mechanism of Rat Dorsal Root Ganglion Neurons on Slope Substrate

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Keywords: Dorsal Root Ganglia (DRG), Axon, Slope Substrate.

Abstract: Neural response to topography depends on the dimensions and shapes of physical features. Most researchers focused on fabricating different grooves and ridges to study cell adhesion, spreading, alignment, and morphological changes. Very few papers report about how sloped substrate influences the behavior of neural cells. In this paper, we made a preliminary experiment to test the reaction of neuronal growth processes to different slopes. We found that all DRG cells' axons couldn't grow across 90 degree slope with 198 μm height. A few axons grew across 90 degree slope with 50 μm height. In addition, we also found that DRG cells showed preference to grow uphill rather than downhill. In future, we will make more detailed experiments to study the mechanism of slope modulation. This study will be helpful for the construction of nerve regenerating scaffolds and neural interface.

1 INTRODUCTION

Every year, thousands of people are disabled by neurological disease and injury. Successful control of the cell behavior will open the ways for neural regeneration and functional rehabilitation. Several therapies offer significant promise for the restoration of neuronal function, including the use of growth factors to prevent cell death following injury (Vincent and Feldman, 2002), stem cells to rebuild parts of the nervous system (Horner and Gage, 2000), and the use of functional electrical stimulation to promote axon regeneration (Al-Majed, Neumann et al., 2000). During these treatments, researchers have found that the interaction of cells with substrate plays a key role in the cell behavior, such as cell adhesion, spreading, morphology, proliferation, and even differentiation. Therefore, the research about how patterned substrates influence the behavior of neurons appears especially important right away.

The first experiment which mentioned the relationship of cells and topography was accomplished in 1911. Harrison (Harrison, 1911) grew cells on a spider web and found the cells followed the fibers of the web. Since then, with the development of micro- and nano-fabrication techniques, a large number of studies have shown that many cell types react strongly to topography. In general, micropattern substrates were fabricated to contain repeating rectangular groove-plateau patterns with varied groove width, varied plateau width and varied groove depth. Neurons follow the discontinuities of grooves and ridges, and attain an elongated shape due to surface-induced rearrangements of the cytoskeleton (Curtis, 2004). Actin and microtubules align along walls and edges, the microtubules being the first element to be aligned, followed by actin (Oakley and Brunette, 1993). Grooved surfaces also induce changes in transcription and the up and down regulation of several genes, but the explicit mechanism for cell guidance has yet to be clarified (Dalby, Riehle et al., 2003).

Most studies focused on the effects of topography size on neurons. Rajnicek et al (Rajnicek, Britland et al., 1997) reported that central nervous system neurites could be guided by shallow grooves with 14 nm deep and 1 mm wide. Stepien and coworkers (Stepien, Stanisz et al., 1999) reported contact guidance for chicken dorsal root ganglion (DRG) neurons on single scratches with 0.1-0.2 mm wide. If the grooves are greater than 20 mm, no cell type (except red blood cells) has been found to respond to the guide (Wilkinson, Riehle et

Li X., Wang Y., Xu Q., Chen F. and He J..
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DOI: 10.5220/0005132600150020
In *Proceedings of the 2nd International Congress on Neurotechnology, Electronics and Informatics* (NEUROTECHNIX-2014), pages 15-20
ISBN: 978-989-758-056-7

al., 2002). Orientation often increases with increasing depth, but decreases with increasing groove width (Clark, Connolly et al., 1990). Goldner et al (Goldner, Bruder et al., 2006) describe an unusual capability of a subpopulation of DRG neurons to extend neurites that spanned across the grooves, with no underlying solid support. The highest numbers of bridges observed under the groove width of 30 mm, even few neurites bridge have been observed spanning a groove of 200 mm.

Although these researches are important for constructing high-resolution neural circuit scaffolds or neural interface, the response of neurons and their axons to the sloped substrata has not been studied in detail. Fricke et al (Fricke, Zentis et al., 2011) constructed a variety of gradient patterns with slight changes in slope to control neuronal cell position, the path of neurite growth, and axon directionality. They found that reduction in the slope of structure from 0.04 (0.3 mm/7.5 mm) to 0.01 (0.1 mm/7.5 mm) strongly decreased the effects on neurite growth. However, because the slope substrate consists of the multiple discontinuous grooves, we can't make sure that slope plays a key role in the difference.

In this paper, we performed a preliminary experiment to test the reaction of neuronal growth processes at different slopes. The purpose of the study was to investigate the preference of axons in growing uphill or downhill on different angles of the slope. Present results of this study can be utilized for nerve regenerating scaffolds or the construction of neural interface.

2 METHODS

2.1 Construction of 90 Degree Slope

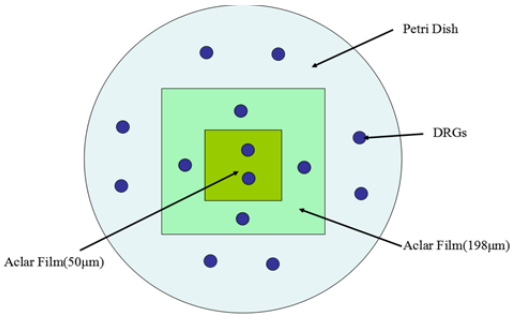

Figure 1: The schematic diagram of 90 degree slope construction with different height. The powder blue disc is Petri dish. The light cyan rectangular is 198 μm thick Aclar film. The green rectangular is 50 μm thick Aclar film. The blue dot is Dorsal root ganglia explants (DRGs).

The scheme of our experiment is illustrated in Figure 1. Two Aclar (Aclar® 33C, Electron Microscopy Sciences Inc., Hatfield, PA) films of different thickness were plated in the Petri dish. The dorsal root ganglia explants (DRGs) were plated on the films or the bottom of Petri dish with different locations. The 90 degree slopes with 50 or 198 μm height were produced in this way.

2.2 Construction of Slight Degree Slope

We constructed an axon stretch-growth bioreactor which contained two independent axon expansion chambers. The axon expansion chamber consisted of a stretching frame that formed a lane, an adjustable towing block that could manipulate cells across the lane, and a projected towing rod for external manipulation (Figure 2).

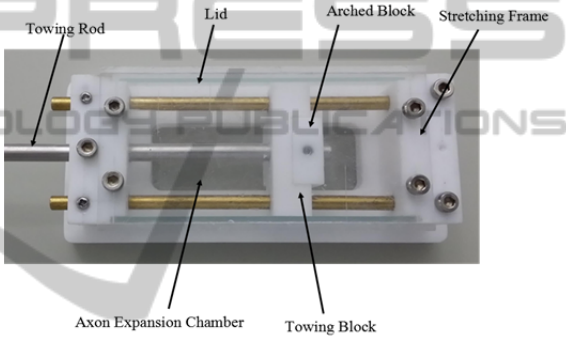

Figure 2: Prototype of the axon expansion chamber. Dorsal root ganglia (DRG) explants can be cultured in the chamber.

The 198 μm thick Aclar film was affixed to the bottom of the stretching frame and spanned the lane. The 50 μm thick Aclar film was held rigidly by the towing block. The 50 μm thick Aclar film was lightly sanded on either side using 1200-grit waterproof sandpaper (MATADOR, Germany) to create gradual slope to the border of the exposed underlying film (Figure 3).

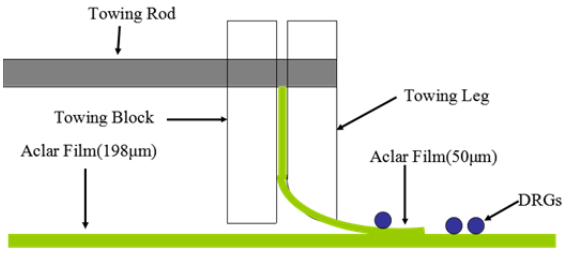

Figure 3: The schematic diagram of slight degree slope construction by axon stretch-growth system. Two different Aclar films (green color) were used: one in the horizontal plane and the other with a slight degree of slope. The blue dot signifies Dorsal root ganglia explants (DRGs).

In order to get more convincing results, we could move the towing rod (Figure 2 and 3) to divide the culture by a computer-controlled micro-stepper motor as described by Smith (Smith, Wolf et al., 2001). This technique results in the stretch-induced growth of fascicular tracts of axons spanning the two membranes. Then we could measure the length and diameter of the regular axons, which provided a measure of the relevant ability of axons to climb the slope substrate.

2.3 Cell Culture

Dorsal root ganglia (DRG) explants were isolated from 1 day infant Sprague-Dawley rats (purchased from the Wuhan University Center for Animal Experiments) as described by Micevych et al. (Chaban, Mayer et al., 2003). The experimental protocol was approved by the Ethics Committee for Animal Research, Huazhong University of Science and Technology, China. Then dorsal root ganglia (DRG) explants were plated in the containers as shown in the Figure 1 and 3. The Aclar films were washed with laboratory detergent, rinsed with deionized water, sterilized in 70% ethanol, and coated with 10 ng/mL high-molecular-weight poly-D-lysine (Sigma, St.Louis, Mo) before the culture.

The culture medium was Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT) supplemented with 10% FBS (HyClone, Logan, UT), 50 ng/ml nerve growth factor (rat β-NGF, R&D, USA), and 1% penicillin/streptomycin. Cultures were treated with the mitotic inhibitors formulated with 10 mM 5-fluoro-2`-deoxyuridine (FdU) (Sigma), and 10 mM uridine (Sigma) to encourage non-neuronal cell elimination. The incubation was conducted at 37℃ in a humid atmosphere containing 5% CO_2. The growth location of DRG explants were observed on the Olympus CKX41 inverted microscope (Olympus Inc. , Tokyo, Japan), and recorded with a Canon 600D SLR (Canon Inc., Tokyo, Japan).

3 RESULTS

3.1 Cell Culture on 90 Degree Slope

After one day of incubation, a few glia cells could be observed by microscope. After three days' incubation, a large number of axons grew out from the DRG explants following the path of glial cells growth (Figure 5-8). Most of the DRG explants were not in the defined position because the Aclar films were shook when we changed the medium. Some growth cones from the DRG explants advanced more than one millimeter after 6 days (Figure 4). However, they couldn't grow down from the Aclar film. Almost all axons and glial cells grew along the edge of 198 μm thick Aclar film (Figure 5).

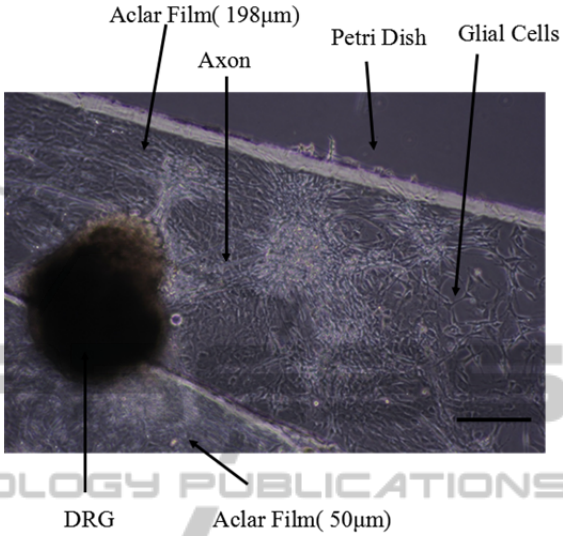

Figure 4: DRG explants cultured in the Petri dish with 90 degree slope. DRG neurite images were taken by a 10×objective, 6 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

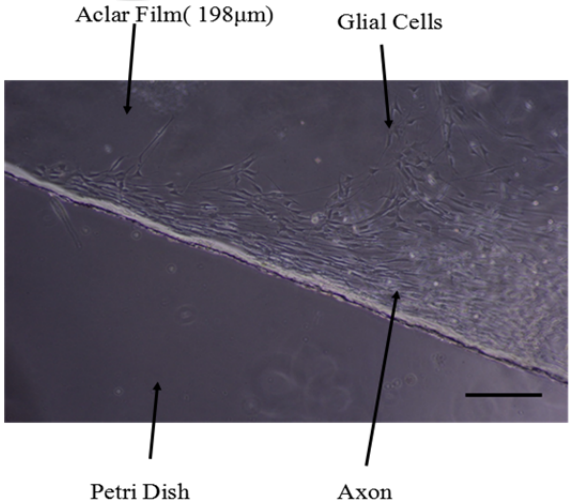

Figure 5: DRG explants cultured on 198 μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 3 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

The morphology of axons grown on the 198 μm thick Aclar film was obviously different from 50 μm thick Aclar film (Figure 4 and 6). Therefore, most axons didn't grow down from 50 μm thick Aclar

film to 198 μm thick Aclar film. A few axons grew across the junction (Figure 7), but we could not make sure that if these axons grew up from the 198 or 50 μm thick Aclar films.

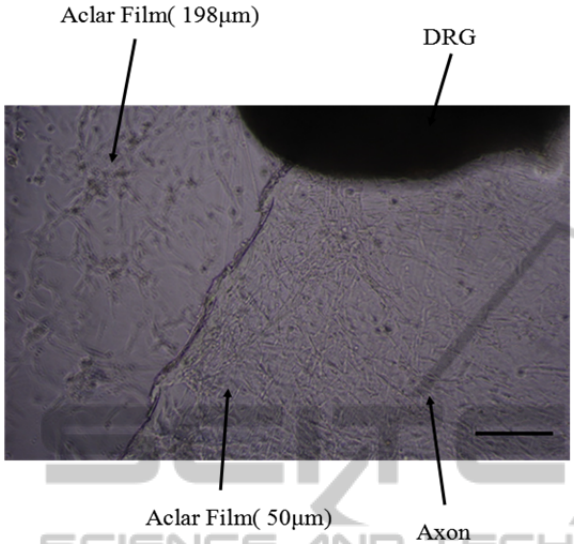

Figure 6: DRG explants cultured on 50 or 198 μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 3 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

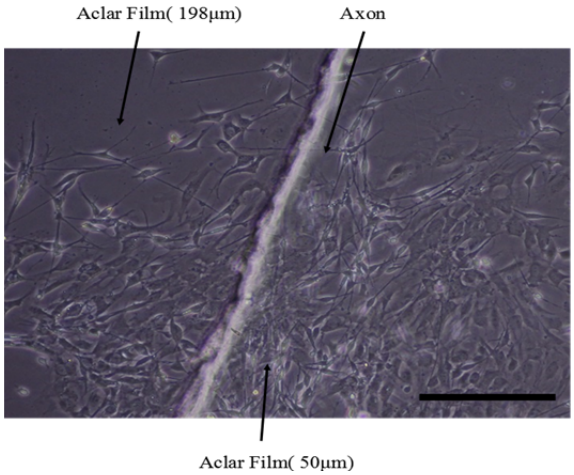

Figure 7: DRG explants cultured on 50 or 198 μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 3 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

On the bottom of Petri dish, a lot of axons and glial cells migrated from the DRG explant (Figure 8). The paths of axons growth were very clear with few glial cells around the DRG explant. However, all DRG explants were too far from the junction with the 198 μm thick Aclar film to fail to arrive at its edge. In order to make sure whether axons can grow up 198 μm from 90 degree slope, we designed another experiment by two pieces of 198 μm thick Aclar film. Finally, we found that it is difficult for axons to grow up on a 90 degree slope (Figure 9).

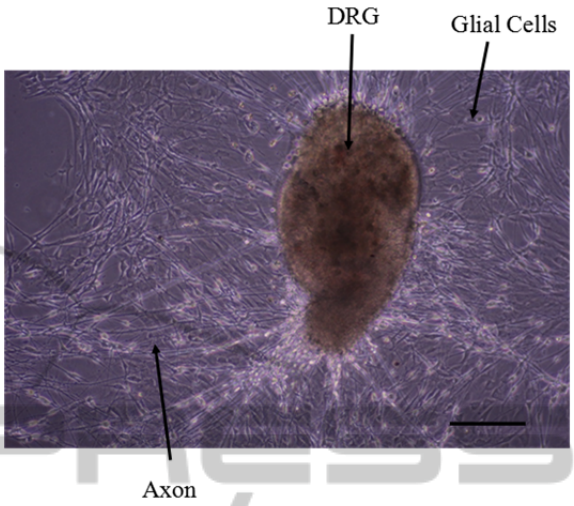

Figure 8: DRG explants cultured on the smooth bottom of the Petri dish. DRG neurite images were taken by a 10×objective, 3 days after the DRG explants were planted in the Petri dish. (Scale bar = 200 μm).

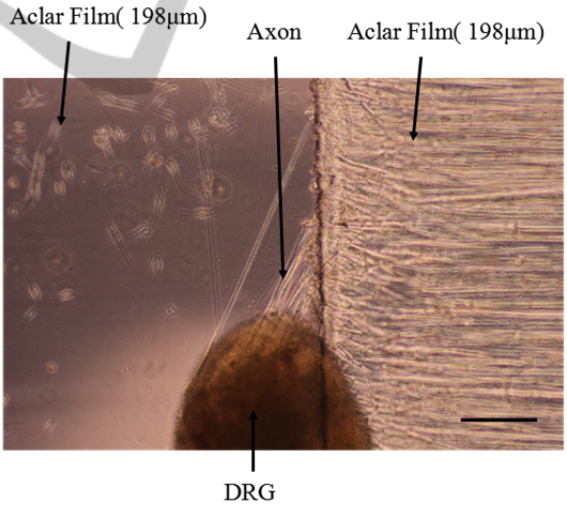

Figure 9: DRG explants cultured on 198μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 3 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200μm).

3.2 Cell Culture on Slight Degree Slope

In the axon stretch-growth bioreactor, a lot of axons grew out from the DRG explants on the 50 μm thick Aclar film (Figure 10). There were more axons on the upper side than lower side. Previous studies have shown that fibroblast cells prefer to grow uphill

rather than downhill (Alaerts, De Cupere et al. 2001). In addition, Johansson et al found the axons of the DRG cells on the original negative (grooved) pattern were always found on the ridge edges, but not in the grooves (Johansson, Carlberg et al. 2006). This may explain why few axons grew down from the 50 μm thick Aclar film (Figure 11).

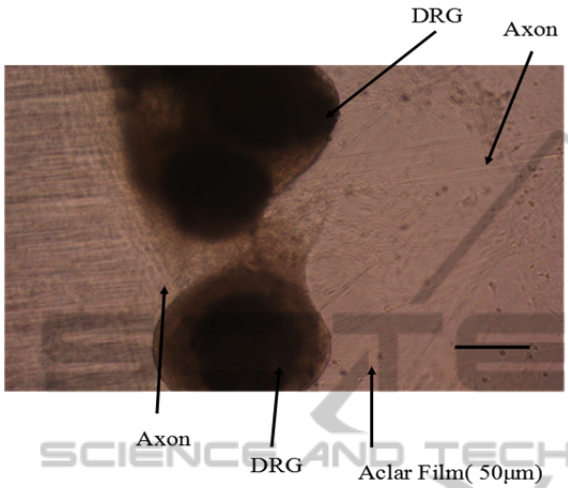

Figure 10: DRG explants cultured on 50 μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 13 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

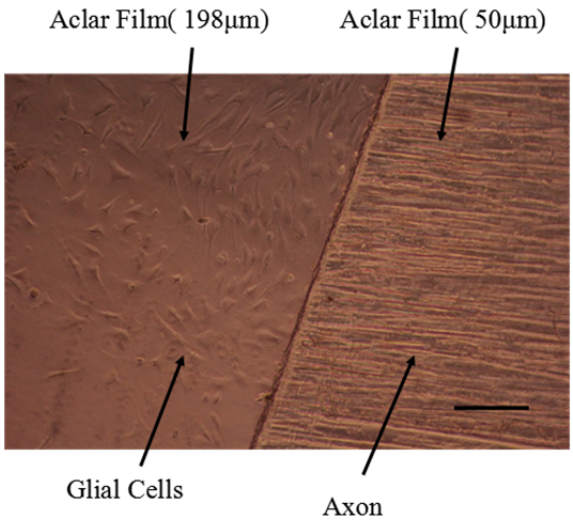

Figure 11: DRG explants cultured on 50 or 198 μm thick Aclar film coated with PDL. DRG neurite images were taken by a 10×objective, 6 days after the DRG explants were planted on the Aclar strips. (Scale bar = 200 μm).

After seven days' culture in the axon stretch-growth bioreactor, only a few of axons extended on the bottom substrate. Stretch was applied by towing the neuronal soma away from the growth cones by taking a series of short 1μm steps. After two and a half days' stretch, the axons grew more than 1.75 mm and showed a regular alignment on the bottom Aclar film (Figure 12). However, the axons didn't cross the 50 μm thick Aclar film, so we could not assess the ability of stretched axons to climb on the slope substrate.

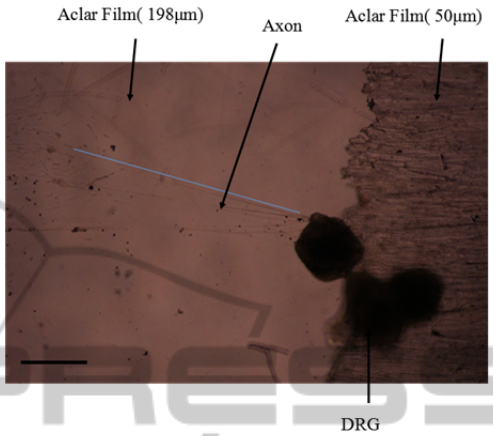

Figure 12: DRG explants cultured on 50 or 198 μm thick Aclar film coated with PDL. The blue line is more than 1.75 mm long. DRG neurite images were taken by a 4×objective, 10 days after the DRG explants were planted on the Aclar strips. (Scale bar = 500 μm).

4 CONCLUSIONS

After nerve injury, neuronal connections are not easily re-established. In the natural environment, neurons are not growing on flat surface but in complex three-dimensional microenvironment formed by other cells or extra-cellular matrix. Along with the chemical signals, neural behavior is also determined by mechanical signals. As scarred tissue is regenerates around the injury site, it is difficult for the regenerating neurites to cross the injury gap. A detailed analysis for the interaction of cells with sloped substrate will not only support the "regenerating axon" to cross the lesion *in vivo* but also be helpful for the three-dimensional neural cell cultures *in vitro*.

In this study, we performed a preliminary experiment to test the reaction of neuronal growth processes on the substrate slopes of different angles. We found that all DRG cells' axons couldn't grow across the 90 degree slope with 198 μm height. A few axons grew across 90 degree slope with 50 μm height. In addition, we also found that DRG cells may also prefer to grow uphill rather than downhill.

This study didn't provide the sufficient details about the mechanisms which actually guide the growth of neuronal cells on the different angles of

substrate slopes, but it was reasonable to assume that the guidance relied on extra-cellular cues, which triggered some reorganization mechanisms in the cytoskeleton. The actin cytoskeleton in cells (fibroblasts, endothelia, and macrophages) reacting to topography is organized in a way which we believe to be appropriate for movement. Some proteins, like semaphorines and ephrins, can inhibit axons to grow the wrong way while other proteins can attract axons to grow on the right way (Cook, Tannahill et al. 1998). Compared with flat surface, growth cones of the growing neurites on the slope would get larger mechanical stress which can affect the strength of the integrin–cytoskeleton links and the integrin receptor distribution and conformation, thus activating intracellular pathways active in cell development and behaviour.

In future, we aim to make more experiments to study the mechanism of slope modulation. For example, we will put DRG explants on different slope substrates to measure the growth rate of neurites and observe the cytoskeleton morphology. Even we will use quantitative real-time polymerase chain reaction (qRT-PCR) to measure the magnitude of changes in the expression of gene compliment which regulates the neuron cell growth on different topologies of substrates. Moreover, neurons are usually not very likely to be the first cells to encounter an implant as any topography may be covered and obscured by glia cells (Franze 2013). Therefore, we also need to study how slope affect the glial cells.

ACKNOWLEDGEMENTS

This study was supported by the Natural Science Foundation of China (grant number 61233015), the National Basic Research Program of China (973 Program) (grant number SQ2012CB037202) and in part by independent innovation fund of Huazhong University of Science and Technology, Wuhan, P. R. China (grant number 2013YGYL004). The authors would like to thank Dr. Jun Ma and Dr. Jianfeng Xu for their instruction in the experiments.

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