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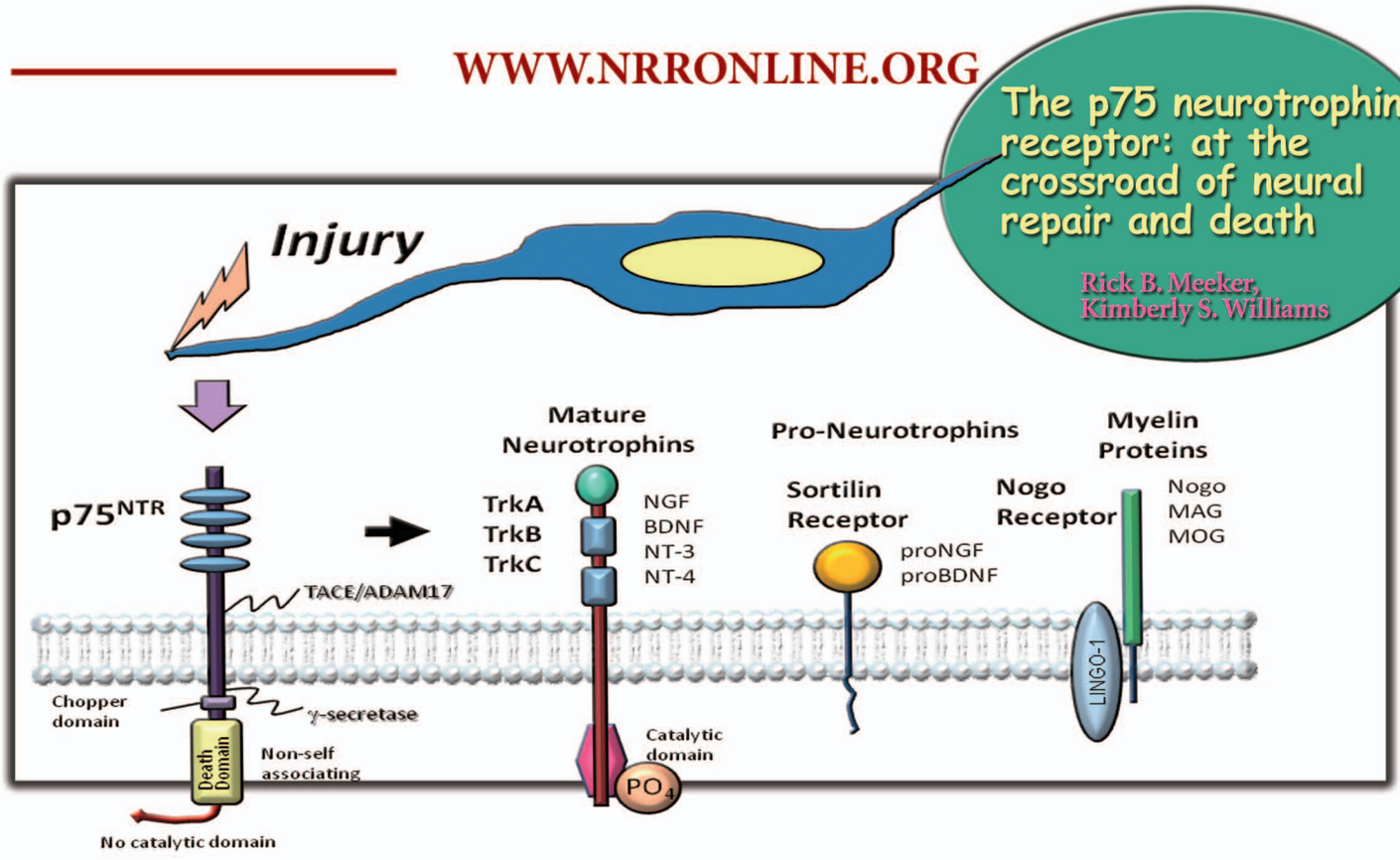

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PERSPECTIVE

New insights on the standardization of peripheral nerve regeneration quantitative analysis

Peripheral nerves form a complex network connecting the central nervous system and the body. Injuries to peripheral nerves often lead to partial or complete loss of motor, sensory and autonomic functions, thus interfering with many aspects of an individual's life.

Despite the spontaneous ability of the peripheral nerve to regenerate, the technical surgical progresses and the significant advances in basic science, the study of post-traumatic repair of peripheral nerves still constitutes a very important biological and clinical issue. The frequently poor outcome reflects the complexity of peripheral nerve injuries and the involvement of several factors during the processes of nerve degeneration, regeneration and target reinnervation. A clear knowledge of the biological mechanisms regulating these post-traumatic phases is therefore a prerequisite to develop innovative regenerative therapies to improve patient's outcome after nerve repair.

Experimental studies of peripheral nerve regeneration are mainly based on quantitative evaluation obtained through 1) functional tests which can vary according to the injured nerve and the animal model (*i.e.*, sciatic functional index, grasping test, ulnar test, skilled paw reaching, computerized gait analysis, reflex latency test, Von Frey test); 2) electrophysiological tests; 3) *in vivo* imaging (ultrasonography and magnetic resonance); 4) nerve fiber morphological analysis; 5) protein and mRNA expression analysis (Geuna, 2015). Among all these techniques, morphological analysis and protein and mRNA expression analysis on the harvested regenerated nerves allow to understand the mechanisms involved in the peripheral nerve regeneration.

Indeed, the complementarity among all these techniques plays an important role in guiding the development of new repair methods.

In order to compare, among different research groups, results obtained on nerve regeneration after different treatments, quantitative data should be obtained. Recent papers (Gambarotta et al., 2014; Ronchi et al., 2014) addressed two important methodological cues for quantitative evaluation of peripheral nerve regeneration, namely the fiber identification during nerve fiber counting and the identification of stable housekeeping genes for mRNA data normalization.

Quantitative morphology analysis: the problem of nerve fiber identification: One of the most important tools to evaluate the success of the nerve regenerative process is the quantitative estimation of myelinated fibers, together with size parameters (axon and fiber diameter, myelin thickness, *g-ratio*).

In the first half of the twentieth century, quantitative morphology was based on light microscopy analysis of paraffin sections, leading to a large variability in fiber counting. After the successive development of resin embedding and the introduction of the unbiased stereological principles, several advancements were made regarding the procedures for the estimation of quantitative parameters (Geuna, 2005; Kaplan et al., 2010). Despite these progresses, the main issue in assessing the number and size of nerve fibers remains their identification at light microscopy level because, especially after injury, the regenerating fibers appear very small.

Because of this observation, a question that arises is whether we are sure to count the total number of myelinated fibers with light microscopy images or if it would be better to use electron microscopy analysis to get more accurate data.

A few studies comparing light and electron microscopy in the quantitative investigation of peripheral nerves were performed already in the 1970s (Eldred and Moran, 1974; Bronson et al., 1978), but the attention was focused only on healthy nerves (third cranial nerve of a rat and femoral nerve trunks of the cockroach *Blaberus discoidalis*).

Given that a huge number of studies dealing with nerve regeneration use quantitative morphology analysis as a parameter to evaluate the regeneration degree, in a recent study the regenerative process was evaluated with a design-based stereological method using both light and electron microscopy (Ronchi et al., 2014). Results showed a significant underestimation of myelinated fiber number quantified with light microscopy compared to electron microscopy, due to the large number of very small axons, especially after nerve regeneration (**Figure 1**). The analysis of the size parameters also showed a higher number of small fibers obtained with electron microscopy analysis. These results were supported by other recent works (Jager et al., 2014; Önger et al., 2014) which also showed a significant difference in the total number of myelinated nerve fibers between light and electron microscopy examinations after peripheral nerve regeneration.

In the light of these results, we believe that the light microscopy is a good starting point for the quantitative study of peripheral nerve regeneration, because it is easier to perform, requires facilities available to everyone and is less expensive. However, if not significant differences are detectable with light microscopy analysis, it may be necessary to integrate and deepen the analysis by electron microscopy to detect any quantitative differences due to the presence of very small regenerating fibers or unmyelinated fibers.

We can conclude that the combination of both light and electron microscopy analysis is fundamental, especially in revealing very small nerve fibers that cannot be distinguishable otherwise. Finally, it is always necessary to remind the discrepancy between light and electron microscopy analysis to be sure not to make mistakes in the interpretation of the obtained results.

Quantitative mRNA analysis: the problem of stable housekeeping gene identification: After injury, different phases occur in the injured nerve: degeneration, regeneration, target

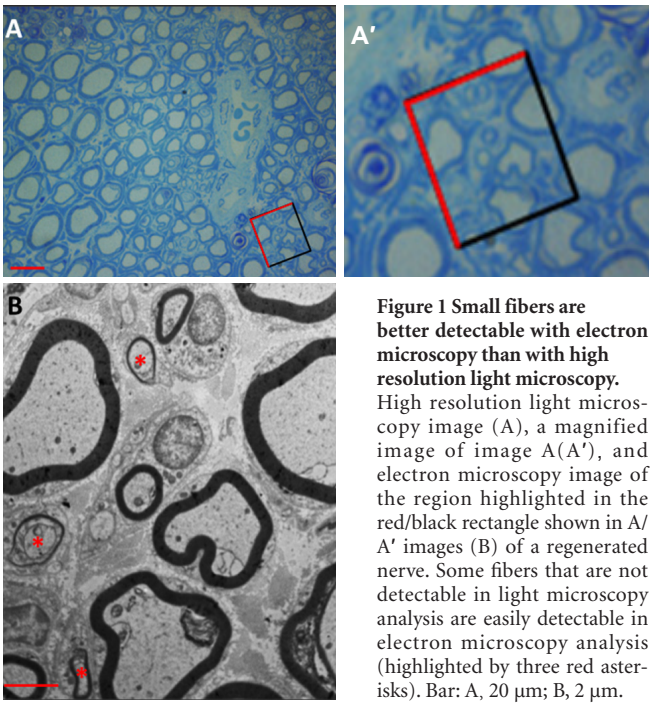

Figure 1 Small fibers are better detectable with electron microscopy than with high resolution light microscopy.
High resolution light microscopy image (A), a magnified image of image A(A′), and electron microscopy image of the region highlighted in the red/black rectangle shown in A/A′ images (B) of a regenerated nerve. Some fibers that are not detectable in light microscopy analysis are easily detectable in electron microscopy analysis (highlighted by three red asterisks). Bar: A, 20 μm; B, 2 μm.

innervations, and remyelination. Dramatic changes happen in terms of cellular composition in the distal part of the injured nerve: Schwann cells dedifferentiate, proliferate and, together with infiltrating macrophages, clean up myelin and axon debris. Subsequently, Schwann cells redifferentiate to remyelinate the regrowing axons. These deep changes of the nerve cellular component are reflected in RNA quality and quantity, making messenger RNA expression analysis very complex. Actually, most of the genes are deeply regulated, including the so-called "housekeeping genes" which are expected to be stably expressed to properly normalize mRNA expression data. Indeed, if the housekeeping gene is down-regulated following nerve injury, the normalized gene will wrongly appear up-regulated, and vice versa, leading to an incorrect interpretation of the results. Therefore, the identification of a gene whose expression does not change in the complex system constituted by the degenerating and regenerating nerve is an important, but difficult aim.

To make evident the fact that the gene expression pattern changes depending on the housekeeping gene used for normalization, in **Figure 2** is shown the expression analysis of two highly regulated genes, namely myelin basic protein (MBP) and neuregulin1 (NRG1) in the distal portion of a crushed median nerve at 3 time points after nerve lesion (1, 7, 28 days), after normalization to 10 different housekeeping genes. This example shows that the up- or down-regulation can be over- or under-estimated according to the housekeeping gene used for normalization, suggesting that it is necessary to choose a criterion to identify the housekeeping gene suitable for the analysis.

Recently, different research groups have addressed the issue of the gene expression normalization in the peripheral nervous system; however, their attention was focused on the spinal cord dorsal horn and on the dorsal root ganglion (Bangaru et al., 2011; Piller et al., 2013).

To identify new stable housekeeping genes to be used for peripheral nerve data normalization, a strategy based on publicly available microarray data obtained analyzing healthy and injured nerves was recently developed. Three independent studies were found, in which normal nerves were compared with injured nerves at different time points; raw fluorescence data were downloaded from Gene Expression Omnibus (GEO) and used to calculate expression fold change (Gambarotta et al., 2014).

Candidate genes stably expressed in the healthy nerve and in the injured nerve at different time points after injury were selected. After the screening, four new candidate housekeeping genes were identified: ANKRD27 (Ankyrin repeat domain 27), Mrpl10 (mitochondrial ribosomal protein L10), RICTOR (RPTOR Independent Companion Of MTOR) and Ubxn11 (UBX domain protein 11).

Gene expression stability was validated by quantitative real-time PCR analysis carried out on RNA obtained from healthy nerves and from the distal portion of injured nerve samples. The stability measure of these genes was calculated using both Norm Finder and geNorm algorithms. This procedure allowed to identify two new and highly stable genes (ANKRD27 and RICTOR) that are useful tools for normalizing gene expression data obtained from health and injured peripheral nerves.

In the light of these results, it can be stated that the relative quantification obtained normalizing data to commonly used housekeeping genes is not suitable. In our opinion, it is necessary to identify 2–3 genes (Vandesompele et al., 2002), whose stability must be carefully verified, and normalize data to their geometric mean.

This approach can be extended beyond the regenerating peripheral nerve: indeed, this study demonstrates that the availability of microarray data can be exploited to identify regulated and not regulated genes also in other fields.

Conclusion: Peripheral nerve regenerative medicine and tissue engineering are hot topics. When different research groups analyze the behavior of the injured nerve following different treatments to promote nerve regeneration, and compare their results, they must be confident that quantitative analyses are reproducible and performed with a shared and standardized protocol.

In conclusion, we can affirm that the standardization of both morphological and biomolecular quantitative analysis is a fundamental step to obtain results to be shared with the scientific community and is an indispensable prerequisite for the good laboratory practice.

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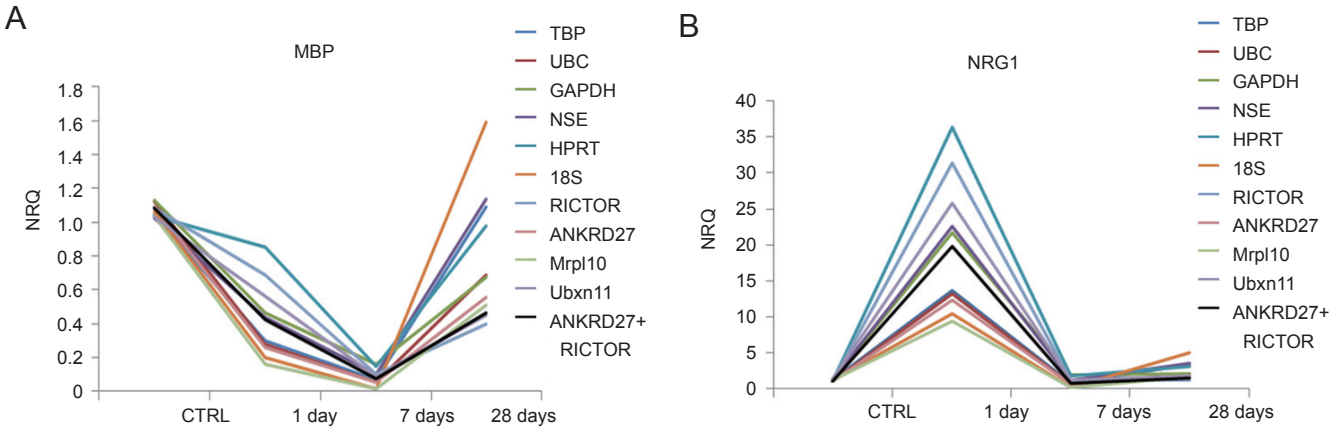

Figure 2 The use of different housekeeping genes affects normalized relative quantification.
The normalized relative quantification (NRQ) of the expression of two genes highly regulated following rat median nerve crush injury (axonotmesis) is shown. Myelin basic protein (MBP, panel A) and Neuregulin 1 (NRG1, panel B) were analysed 1, 7 and 28 days after nerve injury, ($n = 3$ for each time point) on RNA extracted from the nerve segment distal to the lesion site.
Gene expression was normalized to 6 commonly used housekeeping genes (TBP: TATA box binding protein; UBC: ubiquitin C; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NSE: neuron specific enolase; HPRT: hypoxanthine guanine phosphoribosyl-transferase; 18S: 18S ribosomal RNA) and 4 highly stable newly identified housekeeping genes (RICTOR: RPTOR Independent Companion Of MTOR, Complex 2; ANKRD27: ankyrin repeat domain 27; Mrpl10: mitochondrial ribosomal protein L10; Ubxn11: UBX domain protein 11).
This figure shows how the gene expression pattern changes when different housekeeping genes are used for normalization and the gene expression pattern after normalization to the geometric mean of two highly stable newly identified housekeeping genes, Rictor and Ankrd27 (black line).

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