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In vivo Local Protein Synthesis in Peripheral Axons using Click iT Chemistry and Confocal Fluorescence Microscopy.

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Neurons are highly polarized cells with long projections including dendrites and axons. Those are complex cellular compartments where the local protein synthesis (LPS) involving processes like plasticity or regenerative processes[1]–[3]. In peripheral axons most of the LPS studies are performed using *in vitro* models that likely are far away from *in vivo* biology of mature axons. An aspect not yet fully characterized is the fine localization where LPS occurs at mature axons *in vivo*. Only a few studies were made at axons in vivo using radiolabeling techniques, but these methods have multiple risks for the users and the environment and are difficult to perform [4]–[6]-[7].

In recent years new technologies were developed for study protein synthesis by fluorescence microscopy including BONCAT protocols [8]. The principle of the technique is the use of methionine orthogonal amino acids in the cell media that are incorporated in newly synthetized peptides. After fixation steps, those peptides are showed by Click iT Chemistry by the link of a fluorescent molecule to the reactive moiety of the modified aminoacid. In the literature there are many studies that use this technology *in vitro* [9], [10] but not *in vivo*. The present work study the sites of LPS in mature axons of peripheral nerves segments incubated *ex vivo* [13] with the HPG orthogonal amino acid, in combination with Click iT Chemistry and confocal fluorescence microscopy. Our study reveals that optimal fluorescent signal is obtained after 4 hours of incubation with 5µM HPG in DMEM, with significant differences against a negative control using the protein synthesis inhibitor cycloheximide (Figure 1A, 1B, 1C and 1D). In resting conditions signal can be observed inside Schwann cell cytoplasm (Figure 1E) but also in axoplasm regions (Figure 1F) revealing LPS in mature myelinated axons. An increase on protein synthesis eighteen hours after lesion has been reported [7], [11], [12], [13], here we detected an increase of 1.5 fold in the fluorescent LPS signal compared to axons without an injury (Figure 1G, 1H and 1I). The later increase is also consistent with an 1.8 fold increase in the immunofluorescence signal of phospho-p70S6K (Figure 1J), a protein that indicates mTOR activation leading to axonal local translation regulation [14]. Finally, we tested if LPS can be observed in motor ventral root axonal wholemounts depleted of myelin obtained by microdissection [15-16]. We detected HPG positive puncta colocalizing with YOYO-1, a highly sensitive DNA/RNA probe (Figure 2) suggesting that those sites could be site of synthesis at axonal domains.

Taken all together these results support the use of HPG/Click IT Chemistry *in vivo* as a useful tool to study local protein synthesis in mature myelinated axons.

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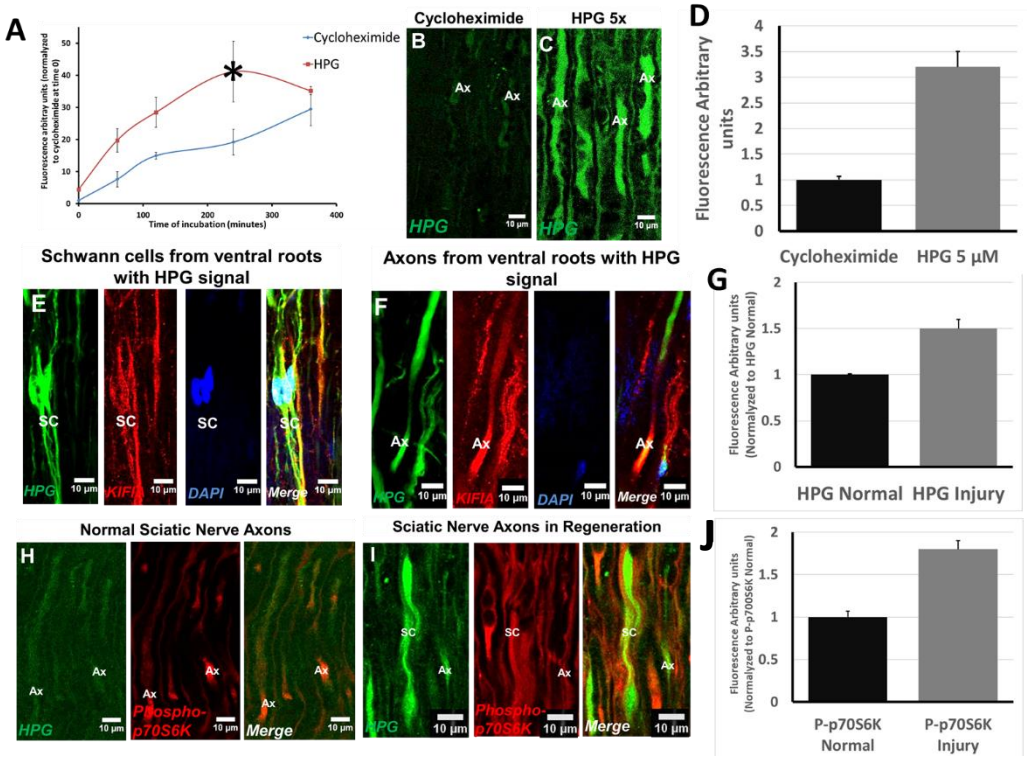

Figure 1. **Distribution of newly synthetized protein signal with HPG labelling in peripheral nerves. (A)** HPG incubation time curve. Asterisk shows the 4-hour (240 min) selected time. **(B)** Cycloheximide control, **(C)** HPG 5 µM selected work concentration **(D)**. **(E)** Schwann cells (SC) & **(F)** Axons (Ax) with HPG signal. **(G-J)** Sciatic nerve axons at normal condition and regenerative state with an increase in HPG and phospho-p70S6K signals. Graphics G and I show the quantification in axons for HPG and P-p70S6K

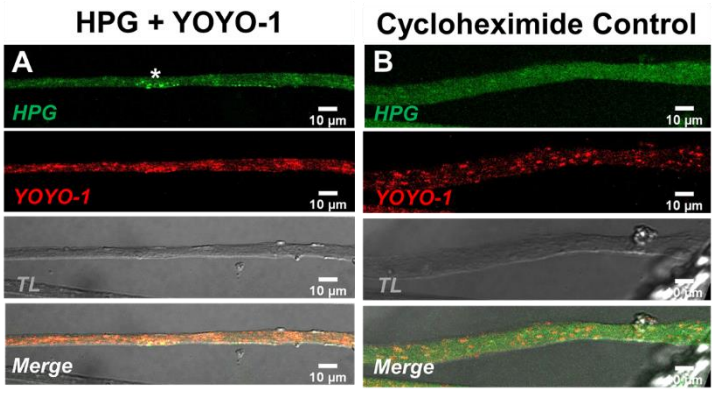

Figure 2. Local protein synthesis signal inside the axoplasm extracted from ventral root nerves. (A) HPG signal co-localized with YOYO-1 signal suggesting protein synthesis domains inside the axon. (B) Control condition with cycloheximide. Transmitted Light (TL) shows the axonal morphology.

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