**In vitro laser scanning confocal microscopy and unsupervised segmentation: Quantification of cytosolic calcium and RNA distribution in hypoxic neurons**

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Abstract— The mimicry of neurodegenerative diseases in vitro can be observed through the induction of chronic hypoxia, and the impact of this stress is monitored using multiplexed imaging techniques. While laser scanning confocal microscopy (LSCM) is a valuable tool for observing single neurons under degenerative conditions, accurately quantifying RNA distribution and cell size by deep learning tools remains challenging due to the lack of annotated training datasets. To address this, we propose a framework that combines 3D tracking of RNA distribution and cell size identification using unsupervised image segmentation. Additionally, we quantified the calcium level in neurons using fluorescent microscopy using unsupervised image segmentation. First, we performed imaging of neuronal morphology using differential interference contrast (DIC) optics and RNA/calcium level imaging using fluorescent microscopy. Next, we performed k-means clustering-based cell segmentation. The results show that our framework can distinguish between distinct neuronal states under control and chronic hypoxic conditions. The analysis reveals that hypoxia induces a significant increase in cytosolic calcium level, reduction in neuron diameter, and alterations in RNA distribution.

Clinical Relevance— The proposed framework is crucial to study the neurodegeneration process and evaluating the efficacy of neuroprotective drugs through image analysis.