Axon guidance is an essential process in neural development and is characterized by cycles of protrusion/retraction of the membrane at the axonal growth cone. Rho GTPases are among the key players in this process; the most studied ones are RhoA, Cdc42 and Rac1. RhoA has been conventionally related to membrane retraction and stress fibers formation, while Rac1 and Cdc42 have been related to protrusions defined as filopodia and lamellipodia formation, respectively. They have been studied in different cell types, yet their activation and spatiotemporal coordination in the rodent hippocampal neurons still awaits further investigation.

In this study, the linear unmixing of Förster resonance energy transfer (FRET) measurements enable us to quantify the fractional activation of RhoA and Cdc42 in lifetime five-minute-videos of respectively 8 and 5 rodent neuronal hippocampal growth cones. Furthermore, two approaches for the quantification of cell edge movements- edge segmentation and line profile correlation- have been developed. The spatiotemporal coordination of RhoA and Cdc42 activity in relation to the protrusion/retraction cycles was measured by defining the cross correlation function between the fractional activation and the edge velocity (the latter was considered positive for protrusion and negative for retraction). Intermolecular FRET biosensors were used for calibration as well as for calculation of the fractional activity. This, the apparent FRET efficiency was reported from acceptor photobleaching experiments to be $E_{\text{D}}=0.35$ and used as a basis to determine the interaction constant between the donor (D) and the acceptor (A); which measured to $K_D=0.003$, $K_D=0.1$ for Cdc42 and RhoA FRET biosensors, respectively. Per-pixel linear unmixing FRET analysis allowed assessing the changes in local FRET efficiency $E_{\text{D}}$, which then could be used to further calculate the fractional activity of RhoA and Cdc42 by excluding the contribution of the fluorescence of non-uniform distribution of free donors and acceptors. We used the edge segmentation computational approach where sampling windows (325 nm width and 216 nm depth into the cell) have been used to extract the edge velocity and the FRET apparent efficiency. On one hand, this method reported in some instances wavy-like movement of the edge; on the other hand, the sampling windows were observed to be laterally moving along the edge which let to false positivity in the extracted results. This was the motivation to further develop the line profile correlation function by drawing a line through the cell edge with sampling windows sliding along this line (325 nm depth and 541 width) and further correlate the extracted edge velocity with the fractional activity of the FRET biosensors.

The findings showed that RhoA activation is synchronous with edge retraction, which supports previous findings in other cell types. Cdc42 reported similar activation synchronous to retraction although with smaller significance. Noteworthy is that Cdc42 showed greater activation than RhoA at 55 seconds prior to protrusion with ($p=0.0246$).