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Live-cell single-molecule localization microscopy-based studies of Cas9-DNA interaction dynamics

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The discovery of CRISPR-Cas9 DNA endonuclease more than a decade ago revolutionized the field of genome engineering. However, a reliable clinical application of Cas9 in the treatment of human genetic disorders and cancer has, so far, been slowed down by such fundamental issues like the bulky size and limited fidelity of this protein. It is very likely that the observed characteristics of interactions between proteins and DNA are completely different *in vitro* versus *in vivo* because of the cell's complex environment. Super-resolution microscopy (SRM) provides the possibility to track individual molecules of a fluorescently labelled diffusing protein of interest in living cells in real time.

Using the miEye – our newly built cost-effective SRM system [1], we performed dSTORM-based single-molecule tracking of *S. pyogenes* Cas9 (SpCas9) labelled with eGFP and HaloTag in live *E. coli* bacteria. We also compared the diffusive behavior of different SpCas9 variants – wild-type, dead, HiFi and others – under the same conditions, as basically all of the previous similar studies were conducted only with the catalytically inactive version of this enzyme (dCas9). To more precisely distinguish the target-bound SpCas9 from the dissociated one, we adapted a switchable multi target-containing biological system enabling the simultaneous co-tracking of SpCas9 and fluorescently labelled plasmid which this protein is bound to.

We further extended our study by performing single-molecule localization microscopy-based *in vitro* characterization of plasma-derived extracelullar vesicles (pEVs) isolated from various animal neurodegenerative disease models. This includes the optimization of a capturing procedure necessary for a robust and integrity-maintaining immobilization of pEVs on the glass surface and establishment of PALM-based imaging of pEVs to reliably determine their size, morphology and distribution in the extracted samples. All in all, SRM-based versatile studies of SpCas9-DNA interaction dynamics *in vivo* and their expansion using a broader approach are efficiently paving the way for the deeper understanding of target search kinetics inherent to this widely-used nuclease.

REFERENCES

[1] M. N. Alsamsam, A. Kopūstas, M. Jurevičiūtė, and M. Tutkus; *The miEye: Bench-top super-resolution microscope with cost-effective equipment*, HardwareX (2022) e00368.