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**Localization of synaptic proteins at nanometer by exTEM & ExR+**

Understanding the functioning of synapses requires an inventory of synaptic proteins at a subsynaptic resolution. However, localizing many synaptic proteins is challenging due to their low expression levels and limited access to immunostaining epitopes. To address this, we have developed the exTEM (epitope-exposed by expansion-transmission electron microscopy) method, which allows in-situ imaging of synaptic proteins with nanoscale resolution [1]. This innovative method combines TEM with size-tunable tissue-hydrogel hybrids to enhance immunolabeling, making epitopes more accessible via molecular decrowding. With exTEM, we have successfully probed the distribution of various synapse-organizing proteins, and we propose that it can be used to study the mechanisms regulating synaptic architecture and function by providing a nanoscale molecular distribution of synaptic proteins in situ. Furthermore, we believe that exTEM can be widely applied to investigate protein nanostructures located in densely packed environments through immunostaining with commercially available antibodies at a nanometer resolution.

Furthermore, we present our recent advancements in fluorescence-based microscopy for achieving super-resolution imaging of synapses. Post-expansion techniques, including MAP, eMAP, and ExR, hold great promise for staining various protein markers in densely packed environments like synaptic clefts. While methods like MAP and eMAP are effective, ExR stands out with a remarkable 20-fold tissue expansion using expandable hydrogel-tissue hybrids. Despite its advantages, ExR has limitations, particularly in antigenicity for low-expression markers, resulting in poor performance in tissues with large expansion ratios (20^3), limiting its application for proteins with low expression due to significant signal dilution. To address this, we have enhanced the ExR protocol (ExR+, tentative) by implementing additional fixation steps for improved protein tethering [2]. Furthermore, we optimized the removal of PFA fixation to enhance the degree of expansion at the dense environments, achieving the high signal-to-noise ratio in images obtained through immunostaining of various low-expression markers. Applications in human brain tissues, have allowed us to investigate human-specific local nanoclusters of synaptic adhesion molecules.

In summary, our integrated approach, combining TEM and tissue expansion for fluorescence imaging, proves to be a successful method for investigating synaptic protein imaging in situ.

**References**

[1] K.-H. Kim, J. Yoon, C.P. Macks, H.-E. Park, J. Youn, J.-u. Lee, M. An, J. Park, J. Ko and C.H. Sohn, *ACS Nano* **2023**, *8*, 9919-9937.

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