APPLICATION NOTE

A1R/AXR Confocal Microscope

3D enhanced resolution confocal imaging of cortical excitatory mouse neurons uncovers dendritic spinule subsets differing in dynamics, regulation, and function

Emerging role of dendritic spinules in synaptic plasticity

Dendritic spines are highly dynamic and changes in their shape and/or numbers can underly synaptic plasticity in cognitive processes, as well as neuropsychiatric diseases. Emerging evidence shows that thin transient protrusions of spines, termed dendritic spinules, can mediate synaptic plasticity in response to changes in synaptic activity. First observed decades ago by electron microscopy (EM), spinules are typically <1 µm in length, finer than filopodia, and most prevalent on large mushroom spines (Petralia et al., 2015). Their nanoscale necessitates resolution beyond the diffraction limit of conventional confocal microscopy. Hence, most spinules studies have utilized EM of fixed brain sections, which provide only a snap-shot in time. Live, time-lapse imaging studies of spinule dynamics are required to better understand their regulatory mechanisms and functions. The early application of super-resolution microscopy for high-speed volumetric imaging has been hindered by the need for special fluorophores, high laser power, lack of speed to acquire Z-stacks, and/or high costs. In this application note, we used strong fluorescence labeling of neuronal elements and optimized Nikon laser scanning confocal imaging parameters, followed by post-acquisition iterative 3D deconvolution using NIS-Elements software (Zaccard, et al., 2021). This technique enabled the visualization and tracking of individual spinules in relation to presynaptic and postsynaptic markers, revealing spinule subtypes that differ in dynamics, length, lifespan, regulation, and function (Zaccard et al., 2020).

Live, time-lapse enhanced resolution confocal imaging of spinule dynamics using resonant scanning

While some prior EM studies have reported spinules emerging from complex perforations in the postsynaptic density (PSD), others have described them originating from nonperforated PSD edges or spine necks (Zaccard et al., 2020). To investigate the origination of spinules in relation to the PSD, we chemically transfected dissociated mouse cortical neurons with plasmids encoding an mRuby cell-fill and GFP-tagged "intrabodies" to label endogenous PSD95 (Gross et al., 2013). The combination of optimized resonant scanning confocal imaging parameters and post image acquisition 3D iterative deconvolution using NIS-Elements software enabled an acquisition speed of ~8 sec/ Z-stack with improved resolution of spinules and PSD complexity (**Figure 1, top**). Our results show that a majority of spinules formed by mushroom spines were exploratory and transient, existing for only seconds, and originating near the edge of simple PSDs (**Figure 1, Lower**). Intriguingly, a long-lived elongated subset of spinules was associated with complex, fragmented PSDs and could traffic PSD fragments (**Figure 1, bottom**). Spinule subsets were differentially regulated by local Ca^{2+} transients, and postsynaptic Rac1-GEF kalirin-7 regulated spinule formation, elongation, and recurrence at the same topographical spine-head locations (data not shown).

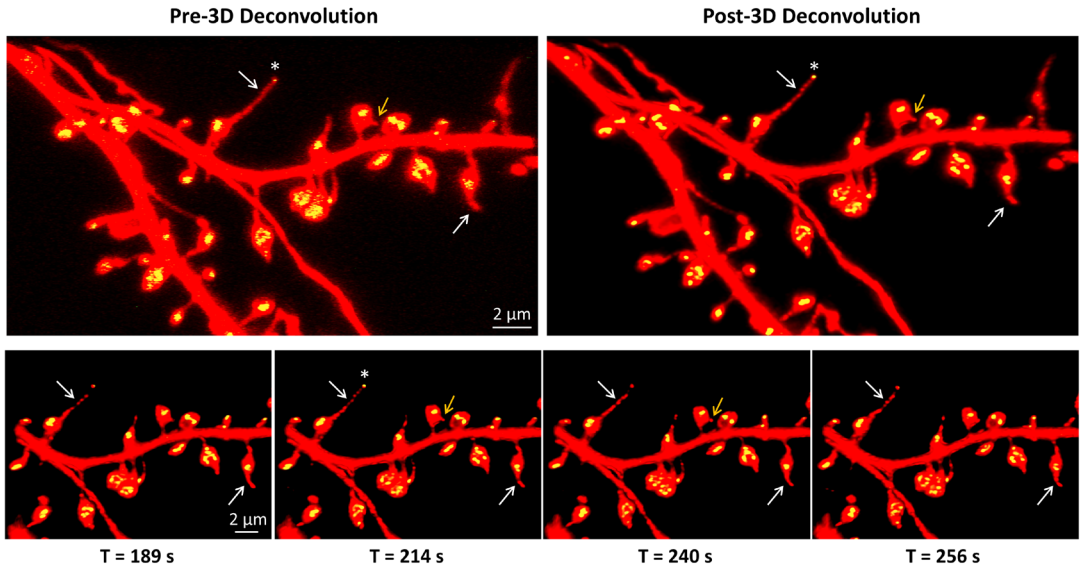


Figure 1. Live, time-lapse enhanced resolution confocal imaging of short- and long-lived spinules in relation to the PSD

Cell treatment: Basal conditions, Green: GFP-tagged anti-PSD95 intrabodies, Red: mRuby cell fill

Plasmids: p-mRuby-N1, Gift from Michael Davidson, Addgene plasmid #54581; pCAG_PSD95. FingR-eGFP-CCR5TC Gift from Don Arnold, Addgene plasmid # 46295

Objective: Plan Apo Lambda 100X, Scanner: Resonant, Line averaging: 4X, Z-slices: 43, Z-interval: ~8 s, Duration: 600 s

White arrows: Long-lived spinules; Yellow arrows: Short-lived spinules; Asterisk: PSD fragment

Enhanced resolution confocal imaging of spinule co-localization with presynaptic terminals in fixed steady state versus activated neurons using galvano scanning

We next performed enhanced confocal resolution imaging of steady state and activated dissociated mouse cortical neurons to investigate the co-localization of spinules with spine-head proximal and distal presynaptic terminals. GFP-transfected neurons were fixed under steady state conditions or after NMDAR-activation for 30 min, followed by immunostaining for the presynaptic marker, bassoon, and phalloidin-647 staining to label F-actin-based structures. The combination of optimized confocal imaging parameters using galvano scanning and line averaging, followed by 3D deconvolution, enabled the visualization of spinules in relation to bassoon-labeled presynaptic terminals and phalloidin-labeled axons (**Figure 2**). Spinules were grouped by length, and the number of proximal and distal presynaptic contacts in basal and activated states were quantified. Our results show that the majority of short spinules (<1 µm) were exploratory and did not contact presynaptic terminals. Activation increased spinule number, length, and frequency of elongated spinule contact with spine-head distal presynaptic terminals. Long, filopodia-like spinules extended to contact multiple distal terminals (**Figure 2, left**), while mushroom-like spinules typically contacted single distal terminals (**Figure 2, right**). Additionally, both presynaptic and postsynaptic markers colocalized to the tips of long-lived mushroom-shaped spinules (data not shown), suggesting the formation of secondary synapses.

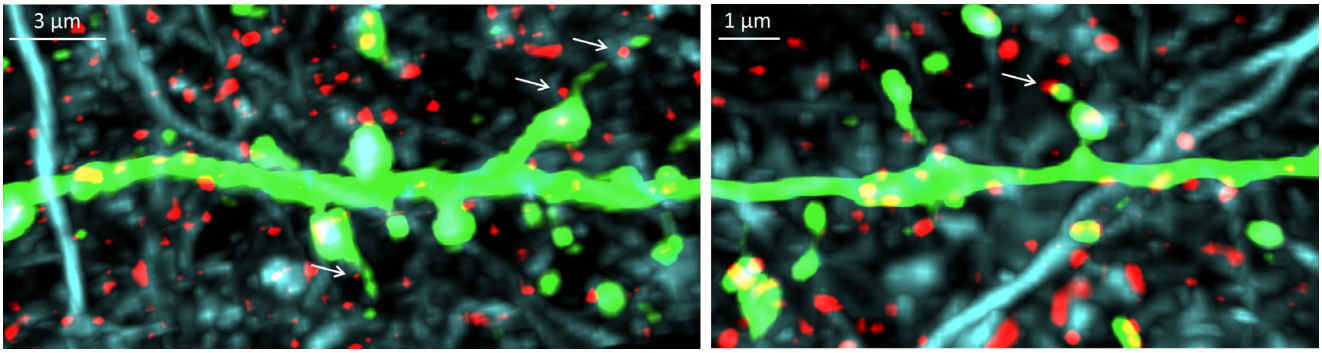


Figure 2. Fixed enhanced resolution confocal imaging reveals the colocalization of elongated spinules with distal presynaptic terminals

Cell treatment: GFP-transfected dissociated cortical neurons were treated for 30 min with media containing APV versus APV withdrawal media, which results in NMDA activation, followed by methanol- free formaldehyde fixation
Green: GFP cell fill, Red: Bassoon, Far-red (appears blue): F-actin Plasmids: pGFP, Gift from Stephen Mayfield, Addgene plasmid # 64904
Antibodies: Anti-bassoon, Guinea pig polyclonal, Synaptic Systems, Cat#141001 F-actin staining: Alexa Fluor 647 Phalloidin, Thermo Fisher Scientific, Cat#A22287
Objective: Plan Apo Lambda 100X, Scanner: Galvano Line averaging: 2-4X, Z-slices Left image: 46, Right image: 34
Arrows: Bassoon-labeled presynaptic terminals co-localizing with spinules

Divergence in dynamics and functions of spinule subsets

Live, time-lapse enhanced resolution confocal imaging of mouse cortical excitatory pyramidal neurons revealed that most spinules emerging from mushroom spines are short-lived, dynamic, and exploratory, originating from relatively near the edge of simple PSDs. Conversely, an elongated, long-lived subset displayed the ability to traffic PSD fragments and form secondary synaptic connections. Quantification of spinules in relation to presynaptic terminals in fixed samples revealed activity-dependent, elongated spinules forming preferential contacts with spine-head distal presynaptic terminals. Elongated spinules developed filopodia and mushroom-like shapes, which mimicked in part the shape and function of dendritic spine classes. These data implicate elongated stable spinules in secondary synapse formation in response to activity, paving the way for future studies on spinule function in the alteration of synaptic connectivity during cognitive processes and/or neuropsychiatric diseases. The basic protocol for enhanced resolution confocal imaging of spinules takes advantage of strong fluorescence labeling, optimized confocal imaging parameters, and 3D deconvolution to visualize fine neuronal sub-structures, e.g., spinules and spine necks, without super-resolution microscopy (Zaccard et al., 2021). The enhanced resolution confocal imaging technique can be widely applied to investigate transient nano-scale biological structures, such as F-actin-based membrane protrusions, in health and disease, without the need for a super-resolution system.

Acknowledgements

Images and text courtesy of Dr. Colleen Zaccard, Research Assistant Professor, Department of Neuroscience, Feinberg School of Medicine, Northwestern University. Images were captured at the Center for Advanced Microscopy/Nikon Imaging Center at Northwestern University.

Reference

1. GROSS, G. G., JUNGE, J. A., MORA, R. J., KWON, H. B., OLSON, C. A., TAKAHASHI, T. T., LIMAN, E. R., ELLIS-DAVIES, G. C., MCGEE, A. W., SABATINI, B. L., ROBERTS, R. W. & ARNOLD, D. B. Recombinant probes for visualizing endogenous synaptic proteins in living neurons. *Neuron*, 2013 Jun 19;78(6):971-85.

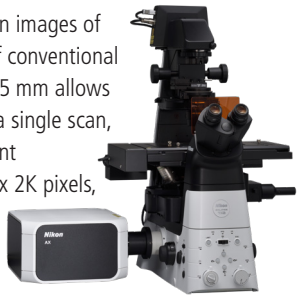
2. PETRALIA, R. S., WANG, Y. X., MATTSON, M. P. & YAO, P. J. Structure, Distribution, and Function of Neuronal/Synaptic Spinules and Related Invaginating Projections. *Neuromolecular Med*. 2015 Sep;17(3):211-40.

3. ZACCARD, C. R., SHAPIRO, L., MARTIN-DE-SAAVEDRA, M. D., PRATT, C., MYCZEK, K., SONG, A., FORREST, M.P. & PENZES, P. Rapid 3D Enhanced Resolution Microscopy Reveals Diversity inDendritic Spinule Dynamics, Regulation, and Function. *Neuron*. 2020 Aug 5;107(3):522-537.e6.

4. ZACCARD, C. R., KIRCHENBUECHLER, D., YOON, S., ARVANITIS, C., & PENZES, P. Protocol for live enhanced resolution confocal imaging of dendritic spinule dynamics in primary mouse cortical neuron culture. *STAR Protoc*. 2021 Apr 5; 2(2):100427.

Product Information

AX/AX R Confocal Microscope



These microscopes achieve high resolution images of 8K x 8K pixels, which is four times that of conventional models. A large FOV with a diagonal of 25 mm allows acquisition of a large area of samples in a single scan, reducing phototoxicity. The AX R's resonant scanner achieves a high resolution of 2K x 2K pixels, allowing acquisition of live sample dynamics with high-speed imaging of up to 720 fps (2048 x 16 pixels).