Neuromuscular junction (NMJ) staining for light microscopy

Neuromuscular junctions can be visualized by light microscopy following labeling of the presynaptic nerve terminal and the postsynaptic acetylcholine receptors. For best results, muscles should be prepared for longitudinal sections, and NMJs in an *en face* orientation can be imaged. In almost all muscles, the endplate band is near the middle of the muscle and this represents the region of interest.

1. Muscles should be lightly fixed in buffered 2% paraformaldehyde (2-4 hours on ice) (*see* **Note below**).
2. The muscles can be prepared for staining in a number of ways, but should be oriented for longitudinal sections.
3. Cutting thick, frozen sections using a cryostat (20-40 µm thick sections), or using a vibratome to cut 50 µm sections of unfrozen tissue (remove the tendons to facilitate sectioning) gives good results. Muscle fibers can also be teased directly on slides to obtain individual fibers.
4. Samples are then stained using standard immunocytochemistry procedures.
5. Presynaptic antigens that work well include a cocktail of anti-neurofilament (for instance the monoclonal antibody 2H3) and anti-SV2 to fully label both the axon and the nerve terminal.
6. The postsynaptic receptors are brightly and specifically labeled using fluorescent conjugates of alpha-bungarotoxin.
7. Standard techniques involving application of the cocktail of primary antibodies followed by washes, then application of a cocktail of the secondary antibody and □-bungarotoxin followed by washes, work well provide patience is used (for instance, primary antibodies should be applied overnight followed by several hours of washing the next day).
8. Standard antibody dilution buffers such as PBS with BSA or normal goat serum as blockering agents can be used, provided generous detergent (0.5-1% triton X-100) is also included.
9. Samples can be viewed on a standard fluorescence scope, but given the large size and 3-dimensional nature of the samples, a confocal Z-series usually gives better results.
10. A number of defects can be readily observed, including partially innervated or completely denervated postsynaptic receptor sites, fragmented or shrunken postsynaptic receptors, atrophied axons or terminals, and swollen or dystrophic axons or terminals. More subtle defects include sprouting nerve terminals and multiple innervation or synaptic sites (single innervation is normally present after two weeks of age). Normal NMJs have a contiguous pretzel-like morphology, the AChR staining has a zebra stripe appearance (the junctional folds), and the nerve terminals completely overlap the receptors. Examples are shown in **Fig. 1**.

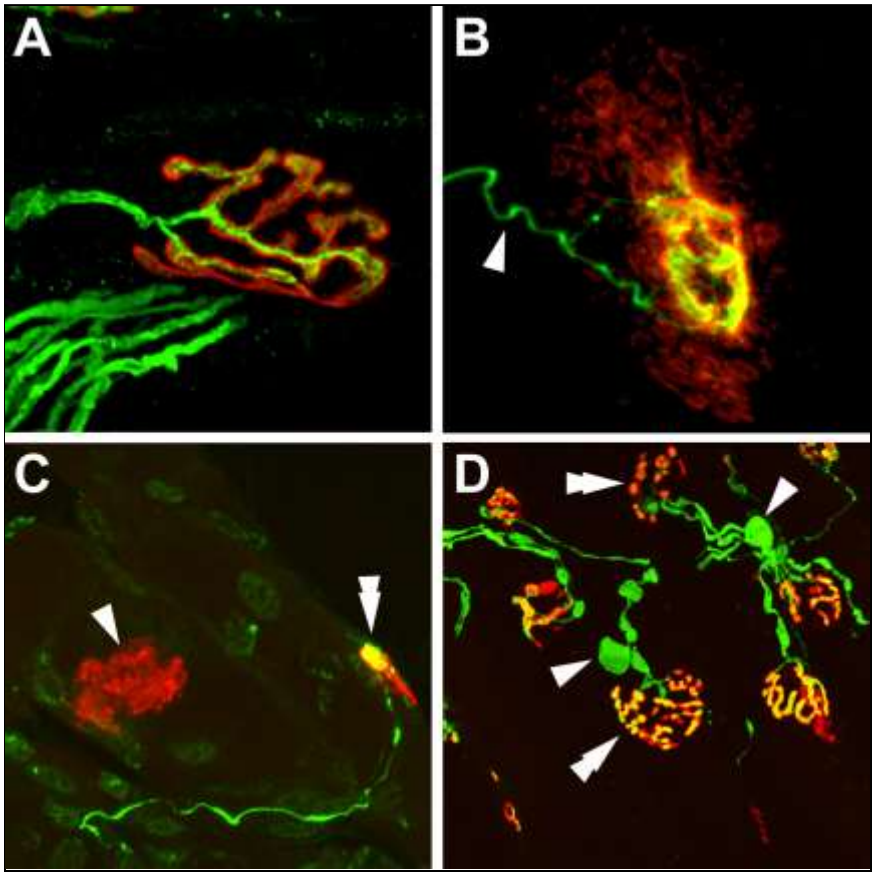

Fig. 1 Neuromuscular junctions. In a control neuromuscular junction (a), the nerve (stained green using anti-neurofilament plus anti-SV2, or using a Thy1-YFP transgenic strain) completely overlaps the postsynaptic AChRs (stained red using fluorescent α-bungarotoxin conjugates). (b) An example of partial innervation, where an atrophied motor axon and terminal fails to completely cover the AChRs on the muscle. (c) An example of frank denervation, where a site of postsynaptic AChRs with no associated nerve (arrowhead) is observed near a site of partial innervation (double arrowhead). (b) and (c) are examples from the GarsNmf249/+ mouse model of Charcot-Marie-Tooth 2D [4]. Other NMJ pathologies are evident in an example from an unpublished spontaneous mutation. The axons and terminals have irregular diameters and varicosities (arrowheads) and postsynaptic sites are fragments (double arrowheads). Such changes are predictive of eventual denervation. These pathologies are also observed in very old mice (greater than 15 months), but are evident in this mutant by 3 months of age

Caveats include minor differences in size and shape from muscle to muscle, and fixation artifacts that can eliminate staining, especially presynaptically. In general, defects in the presynaptic terminal, such as partial retraction, are reflected less-precise definition in the postsynaptic receptors.
Additional analyses that can be informative include staining with anti-S100 to visualize Schwann cells (the terminal Schwann cells play an important role in guiding terminal sprouting and reinnervation (1,2)), and histochemical stains to visualize acetylcholine esterase (3).

Note: *The paraformaldehyde should be of high quality (such as that supplied by Electron Microscopy Services), and should be prepared fresh each day. Overfixation or low-grade fixatives will dramatically reduce or eliminate the antigenicity of presynaptic proteins.*

References

1. Son YJ, Thompson WJ (1995) Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. Neuron 14:133–141
2. Son YJ, Thompson WJ (1995) Schwann cell processes guide regeneration of peripheral axons. Neuron 14:125–132
3. Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EM Jr et al (1998) GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21:317–324
4. Seburn, K. L., Nangle, L. A., Cox, G. A., Schimmel, P. and Burgess, R. W. (2006) An active dominant mutation of glycyl-tRNA synthetase causes neuropathy in a Charcot-Marie-Tooth 2D mouse model. *Neuron* **51**, 715-726.