Circuitry in the Rabbit Inner Plexiform Layer: Enhanced Analysis with Deep-Etch EM Immunogold.

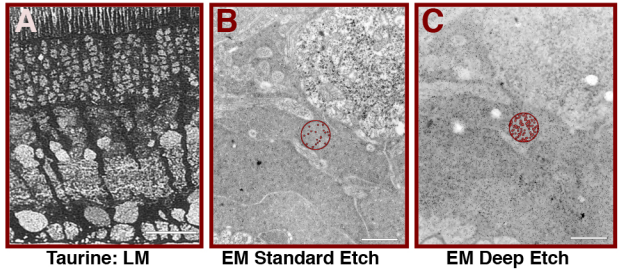
C.B. Watt, J.-H. Yang, B.W. Jones, M.V. Shaw and R.E. Marc. Moran Eye Center, University of Utah

Abstract 2771 B768 Support: NIH EY02576 and Research to Prevent Blindness

Purpose: Our goals were to decipher multiple inhibitory feedforward and feedback circuits that shape ganglion cell receptive fields. We sought to develop high-performance tools for the complete immunolabeling of neuronal and glial cohorts in the mammalian inner plexiform layer by inserting amino acid signals (e.g. GABA, Marc & Liu, 2000, *J Comp Neurol 425:560-582*) into electron microscopic (EM) datasets with the highest possible spatial resolution and signal-to-noise ratios (SNRs) 10-fold greater than traditional EM immunogold (IMG).

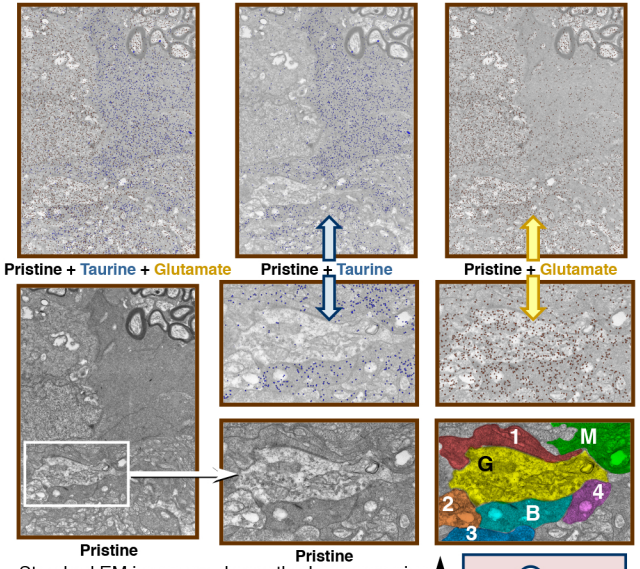
Methods: Ultrathin sections of osmium post-fixed rabbit retina were imaged for pristine ultra-structure. Serial flanking sections were imaged after "deep-etch" IMG amino acid probing and computationally fused to the pristine imagery. Sections on carbon-coated Formvar films were deep-etched with saturated sodium ethoxide. Images were registered with PCI remote sensing code (Richmond Hill, Ontario, CA).

1 Deep-etch increases signal strength 5x

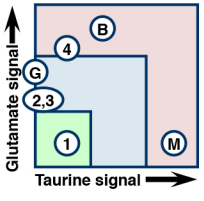


Standard EM immunogold visualization displays lower signal strength and SNRs than LM detection (**A**). Comparison of standard (**B**) and complete **deep sodium ethoxide etching** (**C**) of serial thin sections shows that standard methods fail to detect at least 80% of the available surface epitopes. This 5-fold increase in signal strength improves SNR by 10-fold and spatial resolution by >2 fold.

2 Overlay data sets yield optimal signals



Standard EM immunoprobe methods compromise structure. Optimal fusion of structure and molecular signals is achieved by registration of serial deep-etch data channels to a pristine image. Bivariate taurine and glutamate signatures differentiate Müller (M), ganglion (G), bipolar (B) and 3 types of amacrine cell profiles.



3 Five channel fusion of EM and optical signals

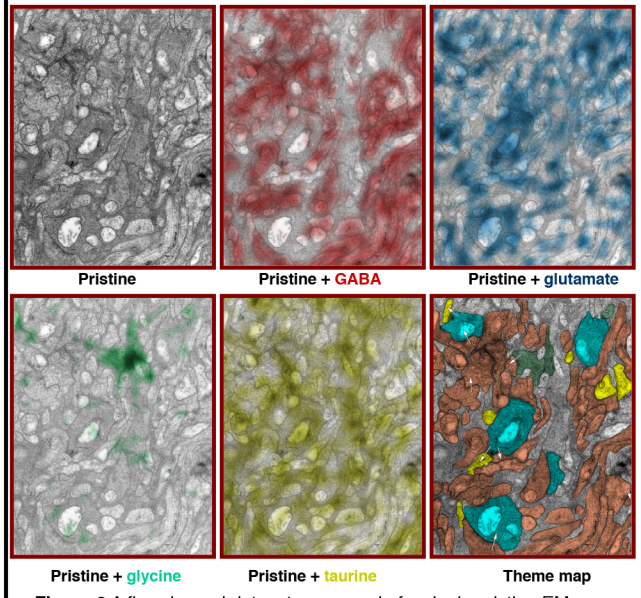


Figure 3 A five channel dataset composed of a single pristine EM channel registered to four optical thin sections probed for GABA, glutamate, glycine and taurine, shown as overlay images above. The theme map codes GABA ACs red, gly ACs green, GCs yellow, BCs cyan, and MCs magenta based on characteristic 4-dimensional signatures. Misalignments often preclude identification of small profiles, but the error is not significantly worse than IMG precision, as optical signal strength is statistically continuous, while IMG statistics are Poisson for small profiles (i.e. <200 nm). However, sampling large areas is much faster with optical overlay as the molecular signatures can be captured by conventional microscopy. This single image documents (1) gly AC > GABA AC, (2) GABA AC > BC, (3) GABA AC > GABA AC, (4) BC > GC and (5) BC > GABA AC transfers validated by molecular signatures.

4 Five channel fusion of EM and deep-etch signals

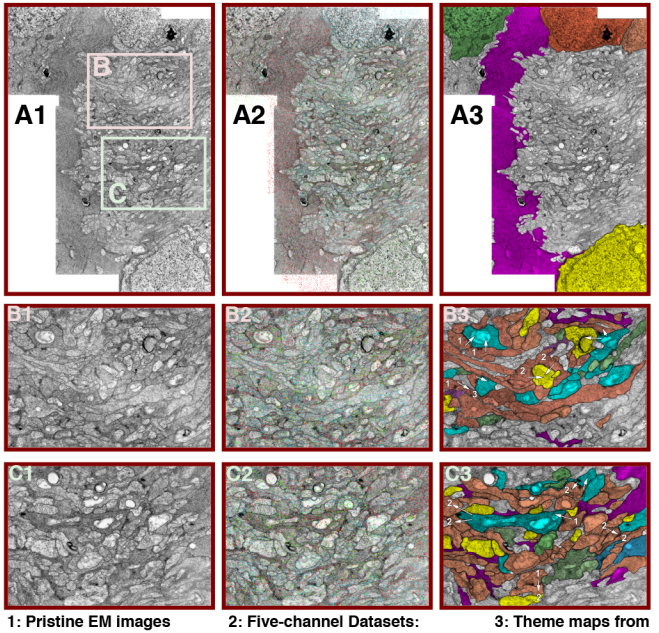


Figure 4 Registration of five images (one pristine and four IMG channels) permits identification of most profiles engaging in synaptic contacts in a routine sample of the inner plexiform layer. Each panel triplet displays (1) the pristine image, (2) the full dataset, and (3) a signatures theme map, coding GABA ACs red, gly ACs green, GCs yellow, BCs cyan, and MCs magenta. (B,C) Enlargements of distal and proximal neuropil containing BC-AC, AC-AC, and AC-GC complexes. Three classes of GABA AC profiles can be defined (1 - high glutamate-taurine, 2 - high glutamate, 3 - high taurine). In panel B3, GABA ACs targeting OFF BC terminals are class 1, while those targeting a GC dendrite are class 2. Panels B3 and C3 both show instances of concatenated inhibitory synapses between different classes of GABA ACs.

Results and Conclusions. **Unlike classic partial-etch IMG methods, deep-etch yields uniform, consistent labeling across entire sections. GABA signals, for example, routinely achieved SNRs of 100:1. Classic methods rarely achieve 8:1, while overlay microscopy often exceeds 1000:1. A near perfect correlation was found between the complete pattern of GABA signals in deep-etch IMG and overlay microscopy, proving that a mixture of methods can capture multidimensional signatures for classification of cell neurites. We demonstrate that individual bipolar cells form synaptic complexes with different signature classes of amacrine cells, including mixed nested and feedforward systems. Deep-etch IMG fused to computational imaging provides superior ultrastructure and amino acid detection. Combined with optical overlay microscopy, multiple signals can be embedded in EM datasets, facilitating circuitry analysis. Almost all inhibitory circuits in the mammalian inner plexiform layer are more complex than the single-stage networks of textbooks and the performance of bipolar to ganglion cell transzfer must be viewed as nested stages of sign-inverting feedback.**