# Label-Free Visualization of Ultrastructural Features of Artificial Synapses via Cryo-EM

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## Methods

### Primary Cultures of Rat Hippocampal Neurons on EM grids

Hippocampal neurons were dissected from Sprague−Dawley rat embryos (embryonic day 17−18) and dissociated to a single cell suspension using a modification of a protocol described previously by Banker (Kaech and Banker, 2006). Neurons were cultured on sterile, PLL-coated Au/Quantifoil EM grids (Electron Microscopy Sciences, Hatfield-PA, USA) together with sterile, PLL-coated coverslips in Neurobasal medium, as described below. Prior to use, the EM grids were immersed in ethanol for 15 minutes and washed several times using Milli-Q water. They were then cleaned with air plasma in a plasma cleaner (Harrick Plasma, Ithaca, NY) in order to remove any remaining carbon contaminants and to make them more hydrophilic. The dissociated hippocampal neurons were resuspended in Neurobasal culture medium supplemented with L-glutamine and B27 at a density of 250,000 cells/mL, and a small drop of this cell suspension was added in a Petri dish containing EM grids and coverslips (6 µL and 80 µL respectively). After 3-4 hours in the incubator at 37°C/5% $CO_2$, 3-4 ml of Neurobasal culture medium was added to the entire dish and the dish was returned to the incubator and kept for 7 or more days *in vitro* (DIV) prior to imaging, while replacing one-third of the medium every 3-4 days. All culture media were purchased from Gibco (Invitrogen). All animal work was performed in accordance with the Canadian Council on Animal Care Guidelines.

### Preparation of Poly-D-lysine coated beads

500 nm silica beads (Bangs Laboratories) were diluted to a concentration of 3 million particles/mL in PBS, washed twice in PBS by centrifugation, then resuspended and incubated in 1mL of PBS containing 0.05 mg/mL Poly-D-lysine overnight at 4 ºC. The Poly-D-lysine treated beads were then washed several times in PBS by centrifugation.

### Coculture with Silica Beads

5µL of the poly-D-lysine coated silica bead solution (sterile PBS, pH 7.4) was added to the neural culture dish directly on top of the TEM grids. The bead/cell coculture was returned to the incubator and left at 37°C/5% $CO_2$ for 24 h prior to freezing of the sample.

**Cryo-EM**

The grids were taken directly from the incubator and were frozen immediately. 5 μL solution of neural culture media was added to the EM grid held by tweezers, blotted, and then frozen hydrated by plunging into a bath of liquid ethane slush (Dubochet et al., 1988). The frozen TEM grids were stored under liquid nitrogen temperature until ready to image. A 626 Single Tilt Cryotransfer System (Gatan Inc.) was used to transfer the EM-grids, which were observed with a FEI G2 F20 cryo-STEM microscope operated at 200KV (FEI, Inc). Images were recorded under low dose conditions on a Gatan Ultrascan 4k x 4k Digital (CCD) Camera System camera at a nominal magnification of 80 x at a defocus level of 2 μm.

Ref:
Dubochet, J.; Adrian, M.; Chang, J. J.; Homo, J. C.; Lepault, J.; McDowall, A. W.; Schultz, P. Cryo-electron microscopy of vitrified specimens. Q. Rev. Biophys. 1988, 21, 129.

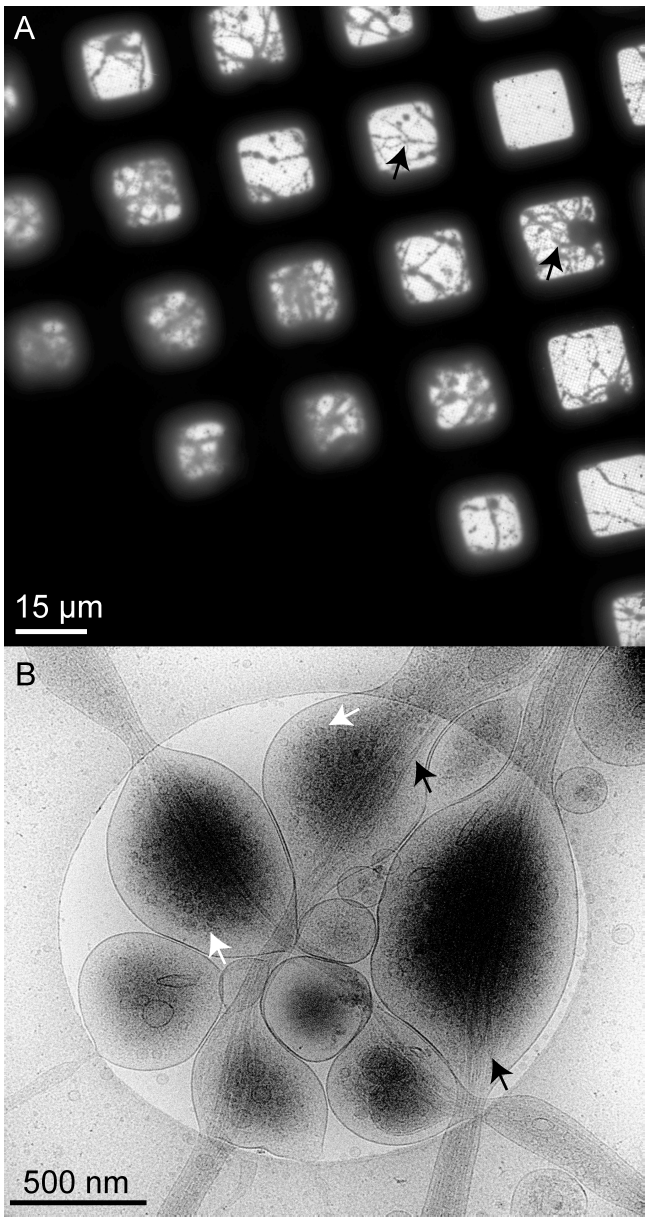


**Figure S.I.1.** Representative cryo-EM images showing hippocampal neurons grown on Quantifoil/Au EM-grids. A low magnification image (A) showing overall neuronal growth on the grid that span over several squares (black arrows). A high magnification image (B) of neurons passing through one of the holes in these squares. Microtubules (black arrows) and synaptic vesicles (white arrows) are visible in this image.

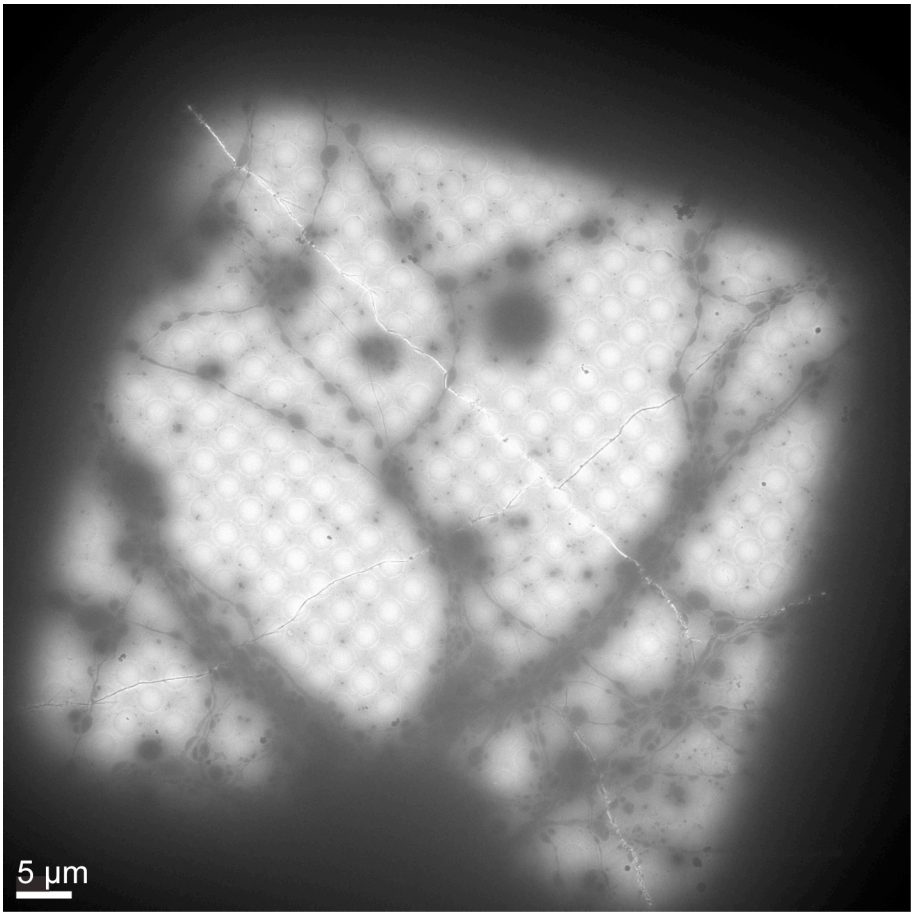


**Figure S.I.2.** A representative cryo-EM image showing hippocampal neurons grown on Quantifoil/Au EM-grids. This low magnification image shows overall neuronal growth on the grid that span over several holes in a single square. As seen in this image, neuronal processes are extending over large length scales.

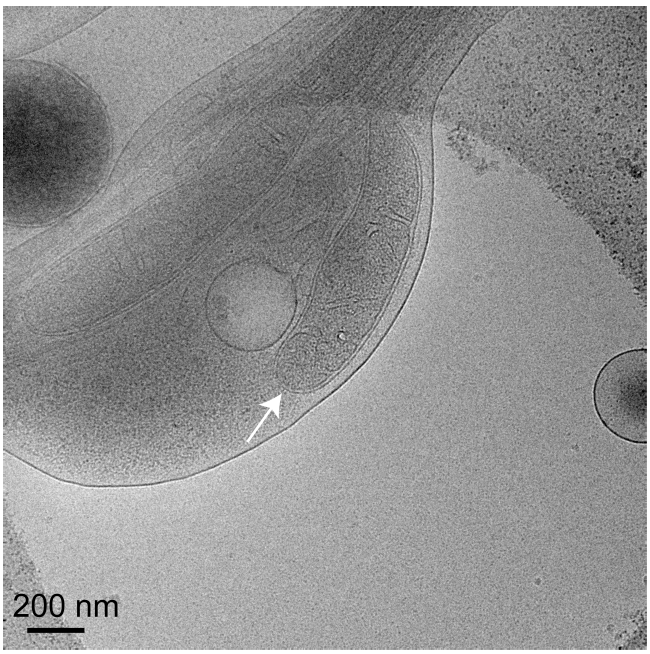


**Figure S.I.3.** Representative cryo-EM image of a hippocampal neuron grown on Quantifoil/Au EM-grids. In this image mitochondria is clearly visible (white arrow).