

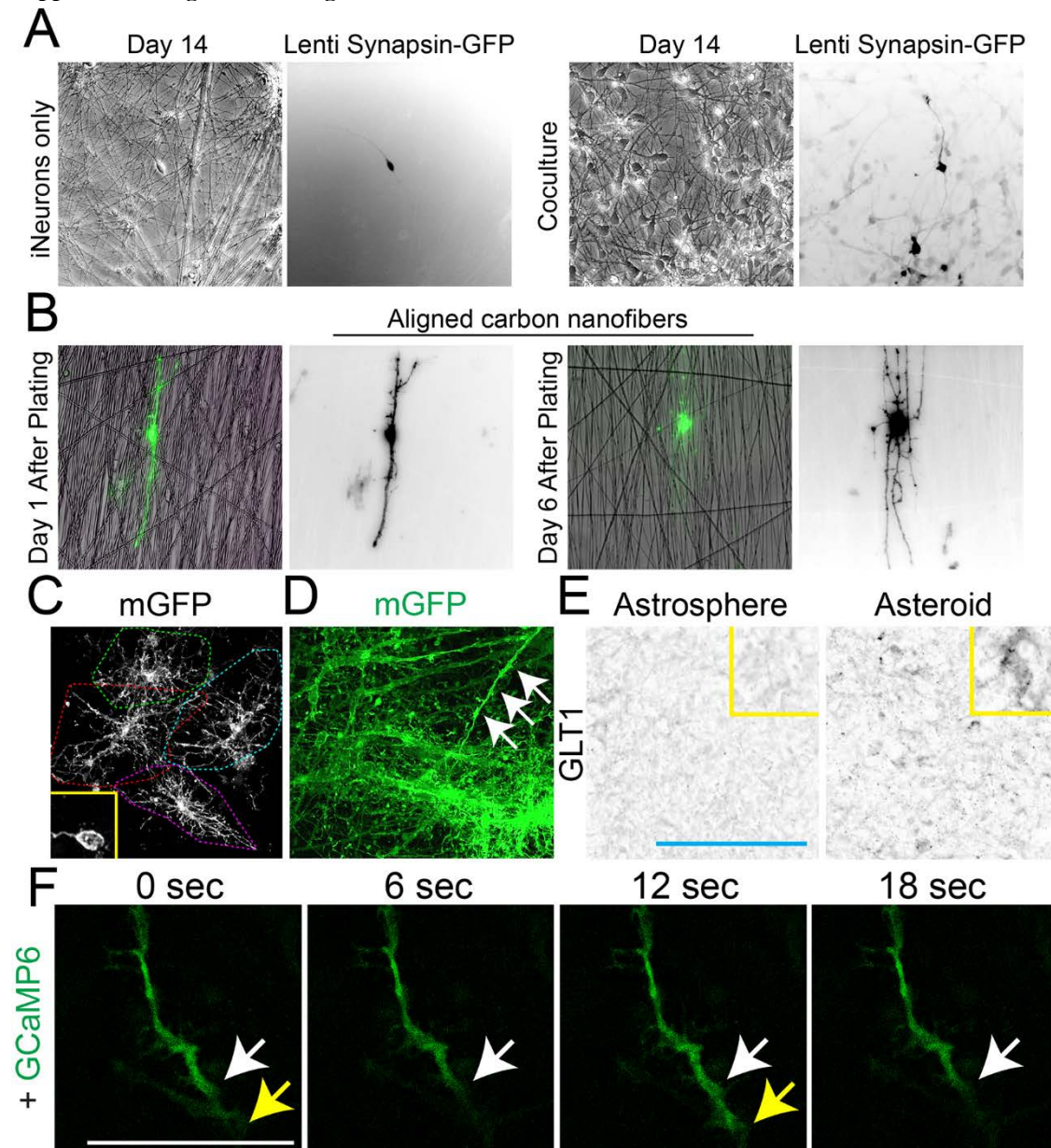
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**Supplemental Information**

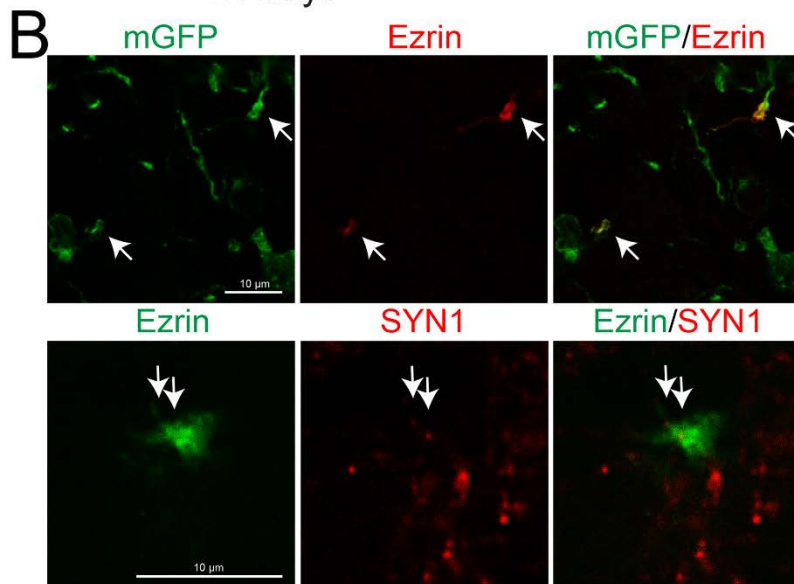
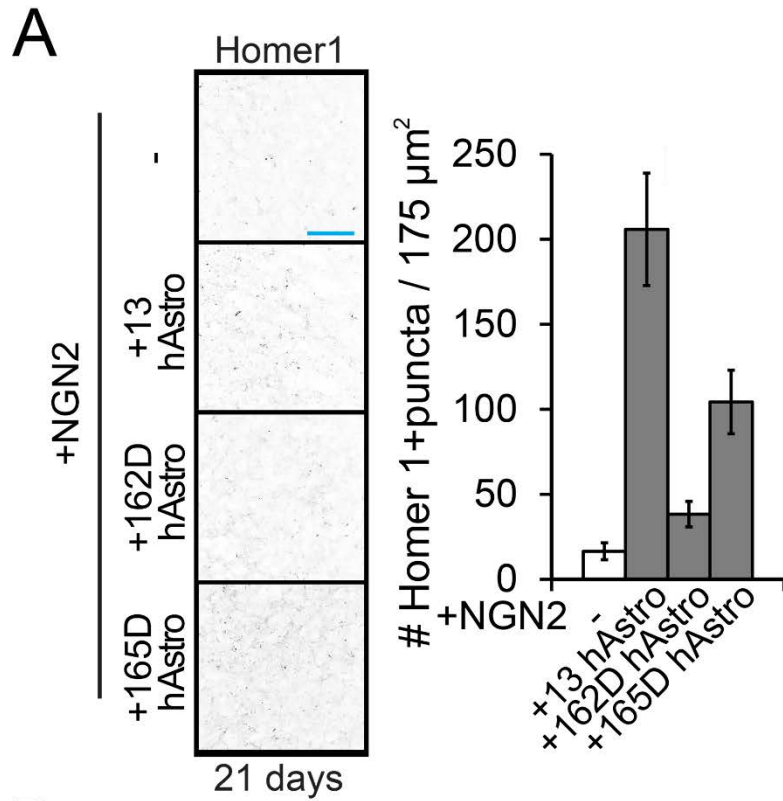
**Systematic Three-Dimensional Coculture Rapidly Recapitulates Interactions between Human Neurons and Astrocytes**

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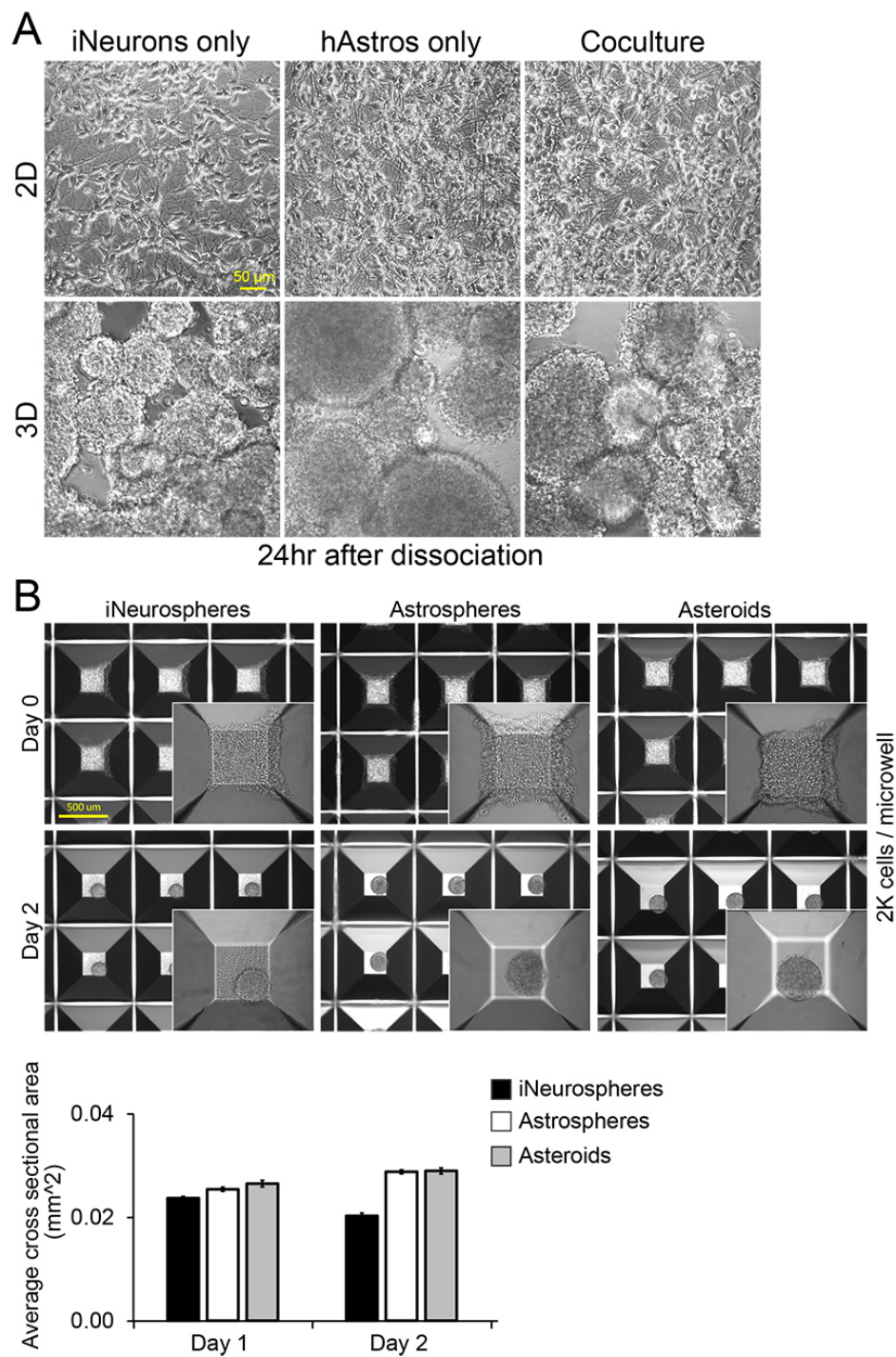
Supplemental Figures and Legends



**Figure S1. Characterization of hAstros in different conditions related to Figures 1-3.** (A) Synapsin promoter activity in iNeurons increases in the presence of hAstros. (B) hAstro branching increases when cultured upon aligned carbon nanofibers. (C) Examples of hAstro territories as shown by manually tracing in different colors. Inset shows an example of bulbous endings that are commonly observed in the different coculture systems. (D) Examples of long processes (arrows) emanating from hAstro somas. (E) GLT1 protein abundance increases on hAstros when cocultured with neurons in the Asteroid system compared to astrocytes alone in Astrospheres. Inset is a magnified view of single soma. (F) Time lapse images of calcium waves (area between arrows indicate the region in which calcium concentrations are changing over time) within GCaMP6-positive hAstro processes in cocultures. Scale bar, 50  $\mu$ m.



**Figure S2. Interaction of astrocytes and synapses within Asteroids related to Figure 3.** (A) Three independent hAstro lines confirm the induction of postsynaptic structures compared to iNeurons only by 3 weeks, yet with variability between lines. Scale bar, 50  $\mu\text{m}$ . N = 6 technical replicate spheres from each group. (B) The perisynaptic astrocyte process marker Ezrin was observed in mGFP-positive astrocyte process endings within Asteroids (confirmed in three independent lines, example from line 162D shown) and closely associated with SYN1-positive presynaptic puncta.



**Figure S3. 3D bioengineering of Asteroids related to Figure 4.** (A) Examples of 2D versus 3D culture methods for pure cellular populations or cocultures. After cell dissociation, cell types were either plated on matrigel coated glass to promote 2D adherence or plated on uncoated glass to allow for random self-organized sphere formation. (B) Micro-well plates allow for the formation of specific-sized single spheres within each well that can be removed for bulk culturing. Individual cells were counted and plated at a density of 2000 cells per micro-well. Size slightly decreased in the absence of hAstros. N = 12 technical replicate spheres per group for area analysis.

**Movie S1.** hAstro extending upon neurons for 2 hr after 24 hr coculture.

**Movie S2.** A single hAstro process from Supplemental Movie 1 in field of view.

**Movie S3.** Timelapse of a GCaMP6-positive astrocyte process within the coculture system. Movie is composed of 70 frames total from images taken every 2 seconds as shown in Figure S1F.