**MATERIALS AND METHODS**

**Multi-electrode array device (addendum)**

The assembloid electrical activity was measured by 8x8 multi-electrode arrays (MEAs) positioned at the bottom of a 12 multiwell. The MEA signals were measured at a sample rate of 12.5 kHz (M768-GLx, Axion Biosystems). A microfluidic system laying on top of the MEA was designed to physically separate the MO and the StrO, allowing their connection only through microtunnels (10 µm width) where neurites can grow along both directions (Figure 4a).

MEA recordings were pre-processed using the AxIS Navigator 3.9.1 software (Axion Biosystems). The spike events were extracted for each electrode using the Peak Detection Adaptive Threshold method, setting an amplitude threshold of 6 standard deviations (Figure 4b). The timestamps of the extracted spikes were exported to MATLAB (2019b, Mathworks) for further analysis (here mention the GitHub File).

**Connectivity analysis (new section)**

The degree of connectivity between different MEA regions was assessed by quantifying the correlation between the recorded spike trains. The Spike Time Tiling Coefficient (STTC, ref. 1) was calculated for each pair of electrodes using the open code repository MEA-NAP (ref. 2). The time interval Δt for the STTC computation was set at 10 ms to privilege the detection of mono-synaptic connections (Figure 4c).

The cross-correlogram provides an estimate of the time delay and signal directionality between electrode pairs. A 0.5 ms time binning was utilized to plot the probability histogram of the time delay (Figure 4d), where the histogram maximum corresponded to the (most probable) time *t* required by the signal to move between the two electrodes, e.g., from A to B. The propagation speed VAB was computed as the ratio of the distance *d*AB (in meters) between A and B and the selected time delay *t*AB (Figure 4d). Only maxima exceeding the histogram mean value by 5 standard deviations were considered as significant. An STTC cut-off of 0.3 was utilized as an additional criterion to accept the existence of a connection between the two electrodes. The signal propagation between A and B was graphically represented as a vector connecting the two electrodes with orientation dependent on the sign (positive or negative) of the time delay. The average signal directionality for the electrode A was computed as the vectorial sum of the vectors connecting A to the electrodes having both significant cross-correlation maximum and STCC (Figure 4e).

The mean directionality of a given assembloid was computed as the vectorial sum of its electrode vectors. During the assembloid development, if the vertical component (y) of the resulting vector reached a value ≥ 2 (i.e. the distance between 2 electrodes), the connection between the MO and the StrO was considered as established (Figure 4f).

For the 92% of the assembloids establishing connection, we calculated the mean vertical (y) and lateral (x) components of their directionality vectors. The vertical component can result positive or negative, depending on the overall direction of the signal from the MO to the StrO or from the StrO to the MO, respectively (Figure 4g). Instead, the lateral component was calculated considering its absolute value without distinction between left and right side of the MEA (Figure 4h).

Ref 1. “Detecting Pairwise Correlations in Spike Trains: An Objective Comparison of Methods and Application to the Study of Retinal Waves”. Catherine S. Cutts, Stephen J. Eglen. Journal of Neuroscience 22 October 2014, 34 (43) 14288-14303; DOI: 10.1523/JNEUROSCI.2767-14.2014

Ref 2. “MEA-NAP compares microscale functional connectivity, topology, and network dynamics in organoid or monolayer neuronal cultures”. Timothy PH Sit, Rachael C Feord, Alexander WE Dunn, Jeremi Chabros, David Oluigbo, Hugo H Smith, Lance Burn, Elise Chang, Alessio Boschi, Yin Yuan, George M Gibbons, Mahsa Khayat-Khoei, Francesco De Angelis, Erik Hemberg, Martin Hemberg, Madeline A Lancaster, Andras Lakatos, Stephen J Eglen, Ole Paulsen, Susanna B Mierau.  *bioRxiv* 2024.02.05.578738. doi: <https://doi.org/10.1101/2024.02.05.578738>