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How we established an *in vitro* Parkinson's Disease model

In this case study, our scientists describe how they established an *in vitro* model of Parkinson's disease – from patient screening, to tissue collection, primary culture derivation, iPSC generation, and even co-culture. Read on to find out how they successfully developed this iPSC-derived assay.

Preclinical models for Parkinson's Disease

Parkinson's Disease (PD) causes progressive degeneration of dopaminergic neurons in the central nervous system (CNS). There is currently no cure for PD, and standard-of-care treatments are limited in their efficacy. New therapeutics are therefore urgently required for this genetically heterogeneous disease.

However, animal testing and 2D models often lack clinical translatability to humans. CNS tissues from real Parkinson's patients are an excellent alternative to these conventional models, as they can be derived from real, human donors affected by PD. At REPROCELL, we strongly believe that human tissue testing is the most relevant way to estimate drug efficacy prior to clinical trial. Unfortunately, fresh CNS tissues are notoriously difficult to obtain, and cannot be procured from living donors.

Alternatively, human neurons derived from induced pluripotent stem cells (iPSCs) can be generated from living patients, improving tissue access and enabling follow-up studies. These iPSCs can be transformed into a range of different neuronal cell types to create co-culture models, further increasing the clinical relevance of PD research.

How we established an *in vitro* Parkinson's disease model

We wanted to develop an *in vitro* model of PD to explore the potential applications in disease research and drug discovery. In this example, we took the model from donor recruitment, all the way through iPSC generation and functional analysis.

Here, we walk you through each step of the establishment of this *in vitro* Parkinson's disease model, including the quality control checks that we carried out at each stage. If you would like to purchase the iPSCs we used to establish this model, they are available on our website.

Step 1: Obtaining Primary Tissues

The generation of iPSCs requires tissues procured from a consenting adult patient. To ensure that our model was as clinically-relevant as possible, we wanted to obtain primary tissues from a donor living with PD.

We located a patient with a sporadic form of the disease who was appropriately consented. A skin punch biopsy was collected from the patient, transported to our labs, and then used to produce a primary fibroblast culture. Below, we have included a phase contrast image of the fibroblasts derived from this PD donor.

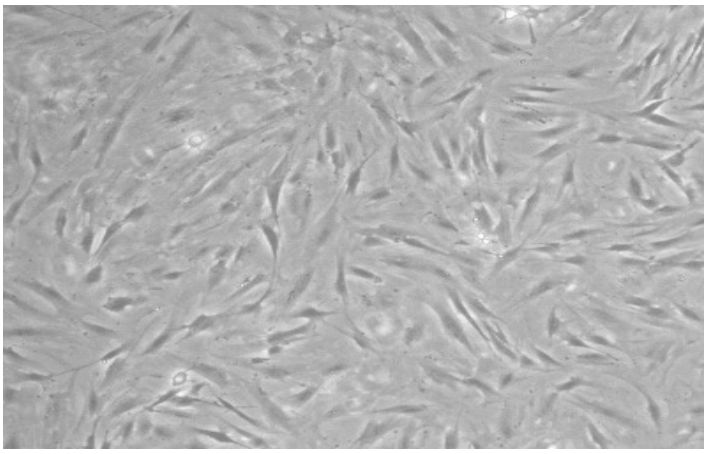

Figure 1: *Fibroblasts derived from sporadic PD patient.*

Quality control procedures we used for these primary tissues

- Immunocytochemistry (ICC) to confirm the presence of fibroblasts in the primary culture (Figure 2A).
- G-banding to confirm normal karyotyping (Figure 2B).
- Genetic profiling via STR analysis.
- Viral pathogen testing, sterility testing, and mycoplasma testing.

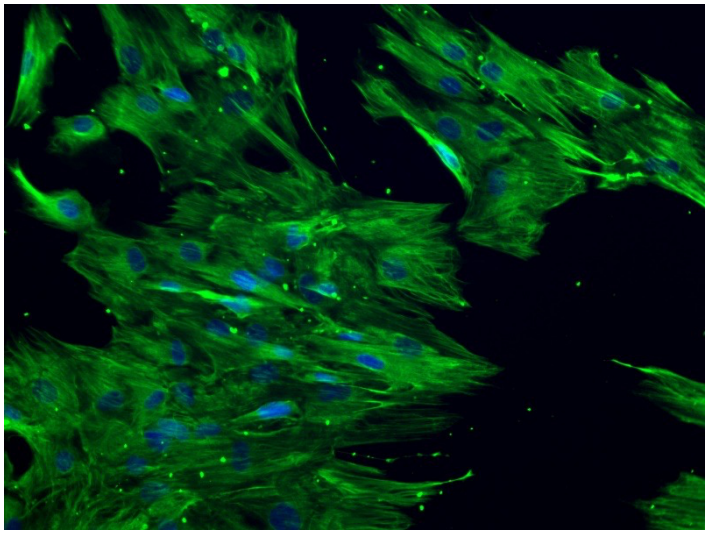

Figure 2A: *Immunocytochemistry (ICC); Vimentin (green); DAPI (blue)*

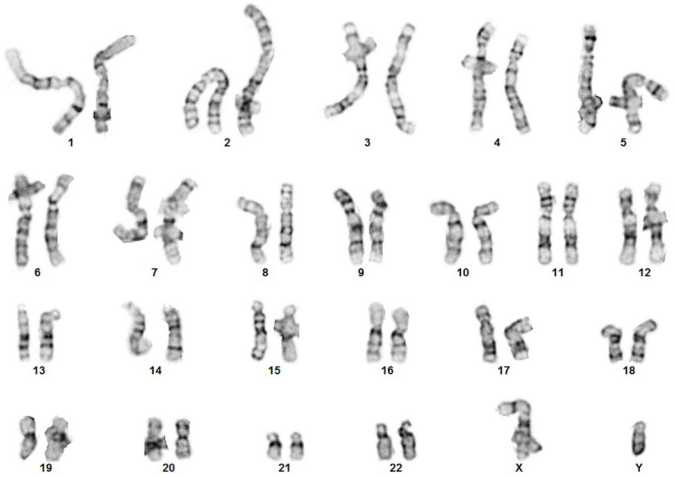

Figure 2B: *Karyotype of the donor fibroblast line*

Regulatory considerations for primary tissues

- Confirm that patients/donors have been properly consented.
- Ensure that the donor clinical data is anonymized.
- Check that the appropriate MTA is in place if primary tissues are obtained externally.

Step 2: Reprogramming primary tissue into stem cells

Following identification of a donor and isolation of the primary culture, we reprogrammed these fibroblasts into iPSCs using our StemRNA 3rd Gen Reprogramming Technology. We chose this methodology as it negates the need to screen iPSC clones, and is 50 times more efficient than other non-integrative reprogramming kits*. The StemRNA 3rd Gen Reprogramming Kit can be purchased from the REPROCELL website with a full reprogramming protocol available here.

We saw the emergence of iPSC colonies just seven days after reprogramming (Figure 3). Quality checks confirmed the potential of these cells to differentiate into any of the three germ layers. These iPSCs also exhibited stronger expression of stem cell markers than the EPCs and iPSC used in the control panel, which we purchased from ThermoFisher.

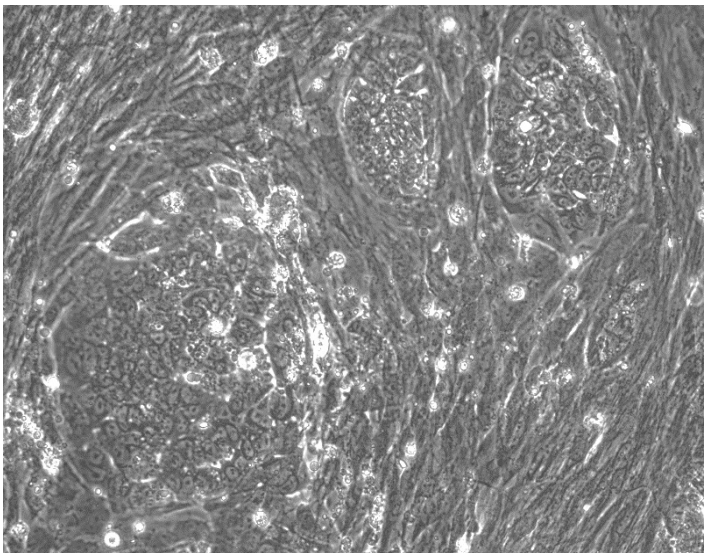

Figure 3: *Using StemRNA 3rd Gen Reprogramming Technology, we saw iPSC colonies emerging a week after reprogramming*

Quality control procedures for RNA-Reprogrammed iPSCs

- ICC of stem cell markers to verify pluripotency e.g. OCT4 (Figure 4A).
- G-banding to confirm normal karyotype (Figure 4B).
- TaqMan hPSC Scorecard Assay to demonstrate pluripotency and trilineage differentiation potential.
- Genetic profiling to check that STR analysis is consistent with the parental fibroblast line.

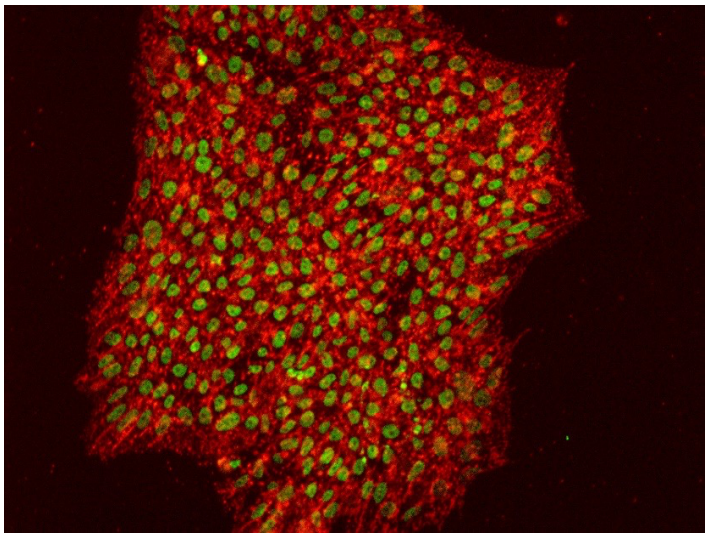

Figure 4A: *Immunocytochemistry (ICC); Oct4 (green); SSEA-4 (red)*

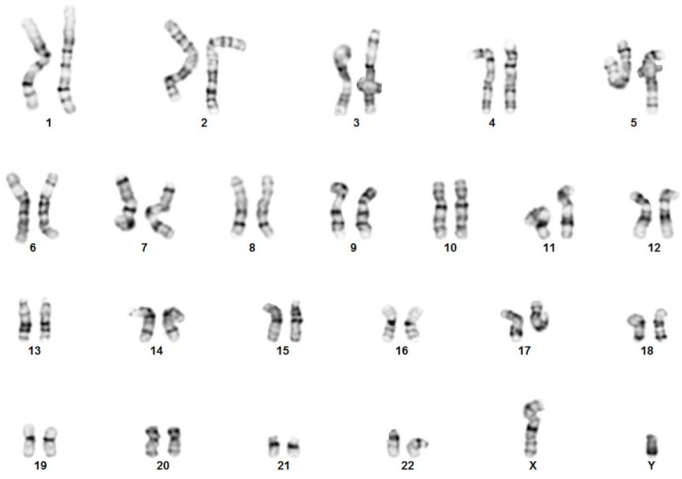

Figure 4B: *G-band Karyotyping*

Step 3: Differentiating iPSCs into Neurons

After verifying the pluripotency, karyotype, and differentiation potential of our iPSCs, we proceeded to differentiate these cells in dopaminergic neurons using a protocol adapted from Kricks et al (2011). This methodology gives rise to dopaminergic neuronal progenitor cells around day 21, and fully mature neurons before day 60.

At day 65, we had produced a homogeneous population of dopaminergic neurons that expressed the neuronal marker Tuj1, and dopaminergic markers TH/PITX3, with very few glial cells present (Figure 5A). Our dopaminergic neurons matured more quickly when cultured in MQ medium compared with astrocyte co-culture or basic differentiation media (Figure 5B).

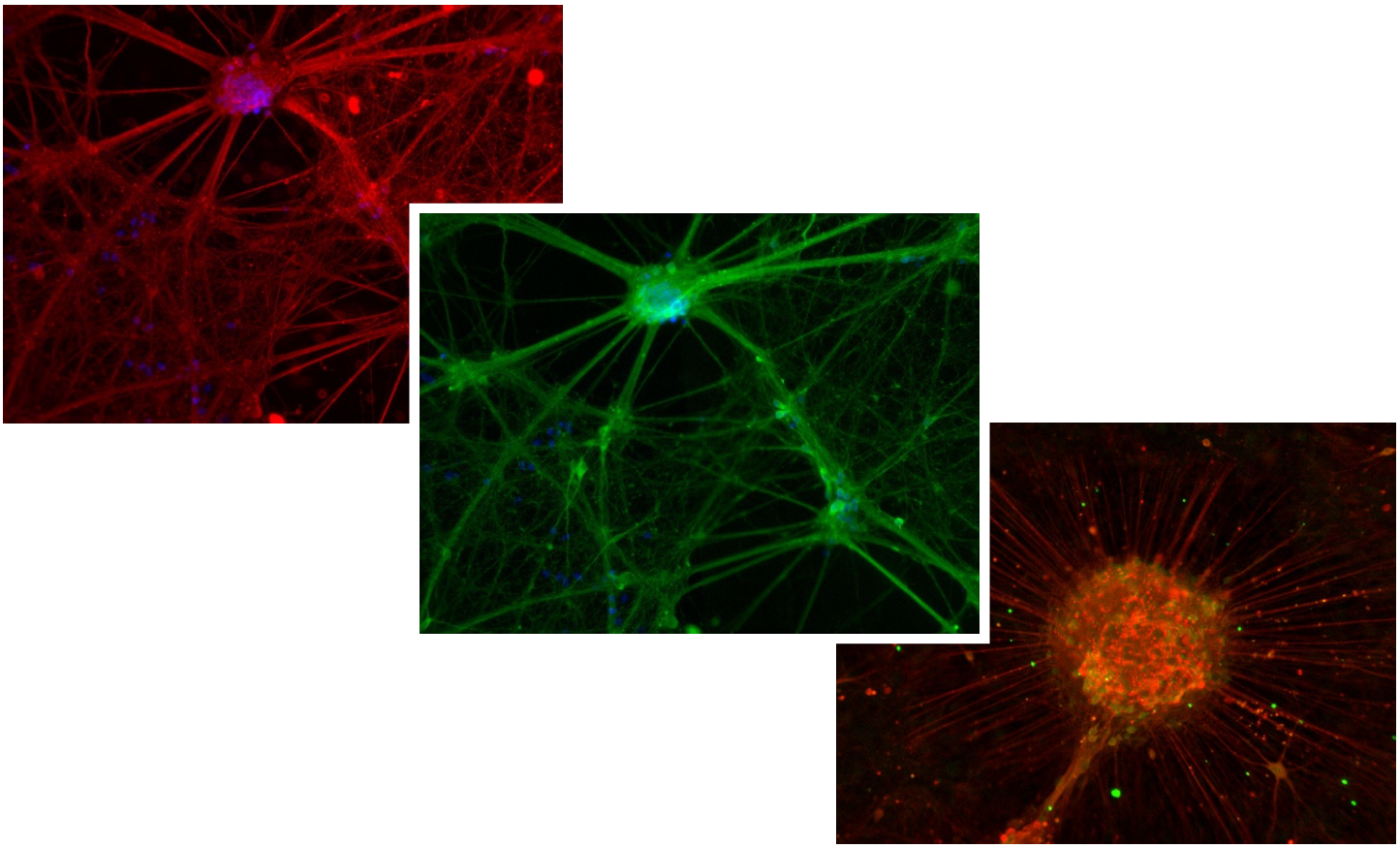

***Figure 5A: Left**: Tuj1 (red); DAPI (blue); **Center**: TH (green); DAPI (blue); **Right**: PITX3 (green); Tuj1 (red)*

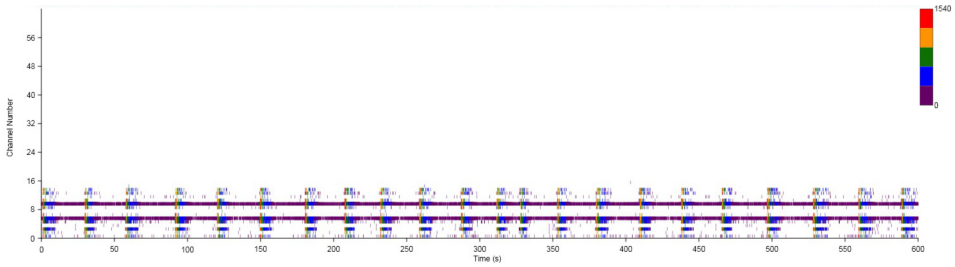

***Figure 5B:** MEA data at week 11 (Neurons 50 days old)*

Quality control procedures for dopaminergic neuron differentiation

- ICC of dopaminergic and neuronal markers e.g. Tuj1, TH, PITX3 (Figure 6A).
- MEA analysis to verify neuronal activity of mature cell networks (Figure 6B).
- RNA seq analysis to check maturity of domaminergic neurons.

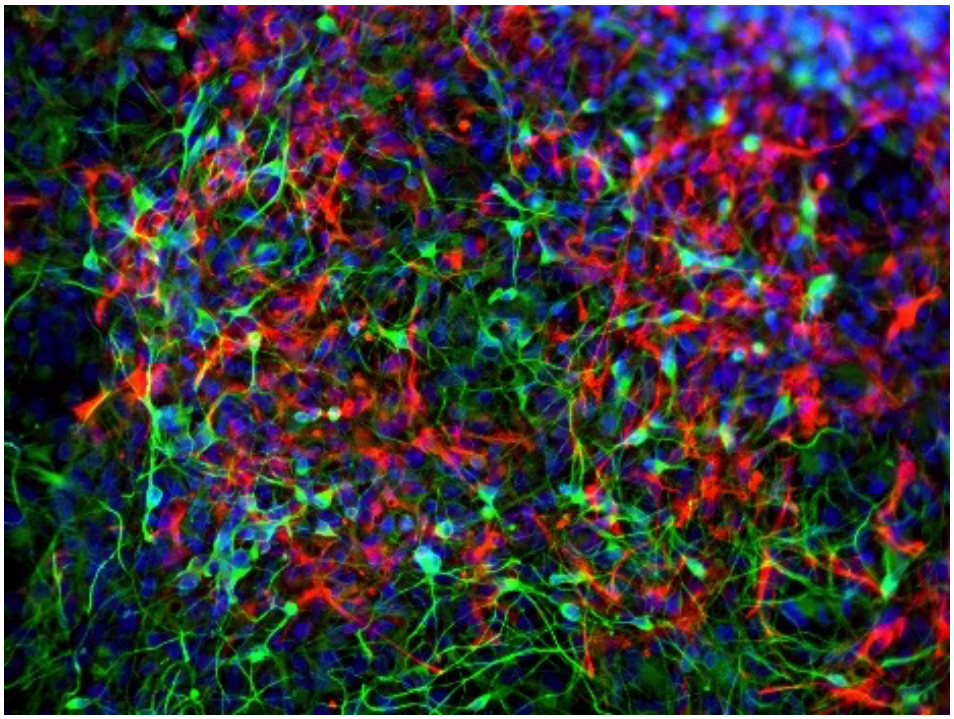

Figure 6A: *Immunocytochemistry (ICC); Tuj1 (green); GFAP (red); DAPI (blue)*

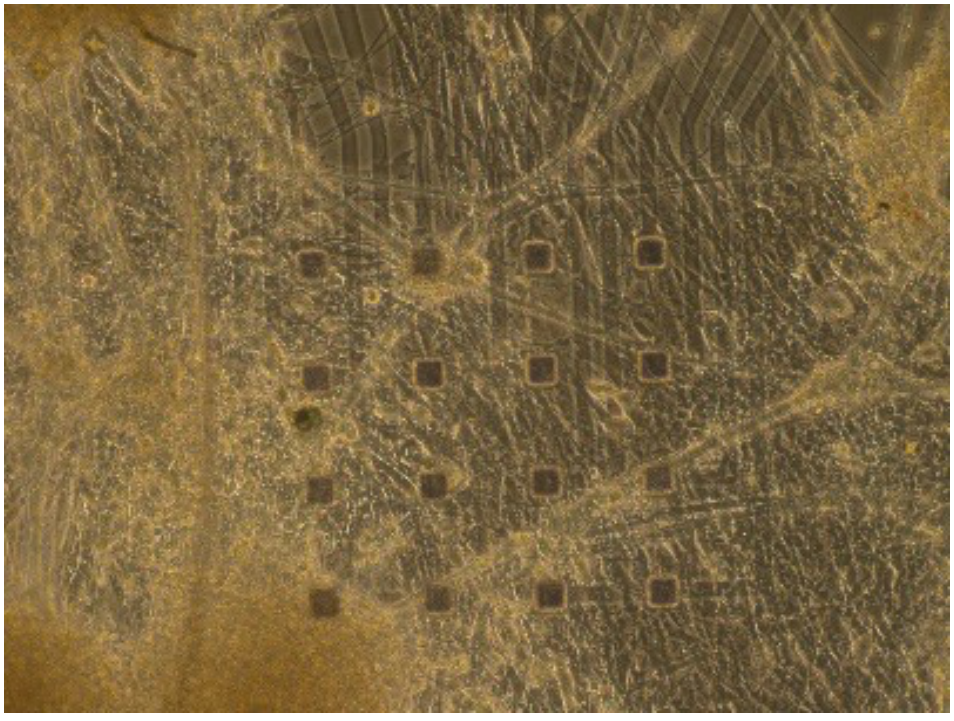

Figure 6B: *Image of mature neuronal network plus microelectrodes*

A deeper look at culture optimization and analysis

Once we had successfully derived dopaminergic neurons from our iPSC culture, we moved onto optimizing the culture conditions for neuronal maturation. It was important to show that the neurons had matured successfully, but also that they displayed functionality.

We used a range of analytical processes to ensure out culture conditions were optimal, including RNA seq and Micro-electrode array (MEA) analysis. The three culture conditions we explored for our neurons included:

1. Basic dopaminergic medium
2. MQ medium
3. Co-culture with iPSC-derived astrocytes (802-3G)

Co-culture of neurons with astrocytes

To produce the co-culture system, we had to differentiate a second control line into astrocytes. These cells were matured until day 95 where their astrocytic identity was confirmed with GFAP and CD34 staining.

However, we soon realized that a physical co-culture would not be possible for RNA seq analysis, as a pure dopaminergic neuron population would be required. We therefore cultured the astrocytes in a trans-well above the dopaminergic neurons, which allowed us to compare culture conditions without the interference of glial cells.

RNA seq analysis results

When we looked at the result of the analysis (Figure 7A), it is striking that the genetic background of the cell line is a bigger factor for differences in gene expression than the culture conditions. We found that the number of genes in common is extensive with only less than 4% being divergent (Figure 7B).

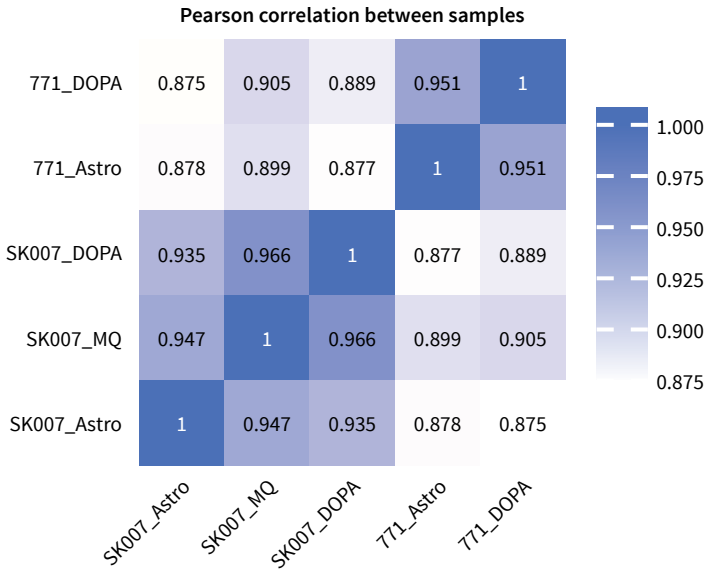

Figure 7A: *Pearson Diagram. The genetic background (control vs PD) is a bigger factor than the method of maturation (control medium vs co-culture).*

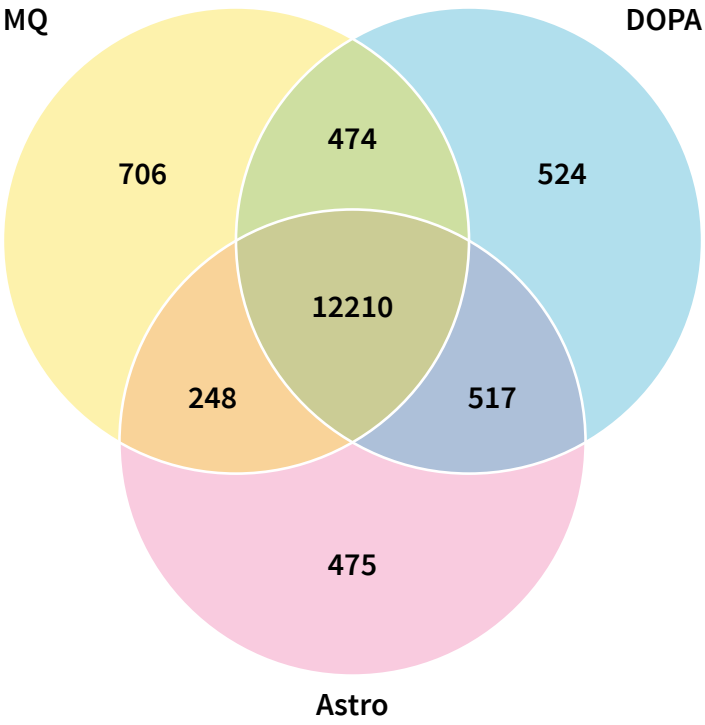

Figure 7B: *Venn diagram. The number of genes in common between culture conditions is extensive (less than 4% are divergent).*

Micro-electrode array analysis results

In preparation for MEA analysis, we plated our freshly differentiated neurons onto wells that each contained 16 electrodes. A true astrocyte/neuronal co-culture could be used for this experiment, meaning no transwells were required.

MEA recording was performed every seven days after the initial plating for a total of 12 weeks. During the first few weeks, no spontaneous neuronal firing was recorded in any of the culture conditions. After week three, we noticed an increased electrical activity in the wells containing MQ Medium and those with the co-cultured cells, but not all electrodes were firing (Figure 8A).

The results at week eleven were striking. While the co-cultured neurons displayed strong electrical activity, no synchronicities were present. Cells cultured in MQ medium displayed strong electrical activity AND the spikes were synchronous in the majority of electrodes. This is a phenomenon called bursting, which is characteristics of mature functional, neuronal networks.

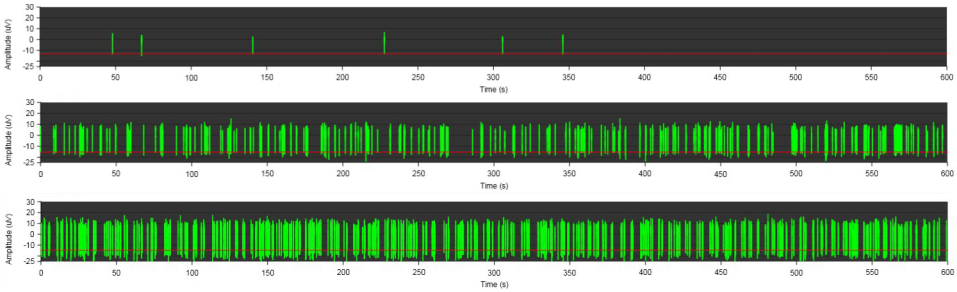

Figure 8A: *MEA data at week three (raw data). Each green line represents a spike of activity over time, and each graph represents the recording of one electrode over time.* ***Top****: Dopaminergic medium;* ***Middle****: MQ Medium;* ***Bottom****: co-culture.*

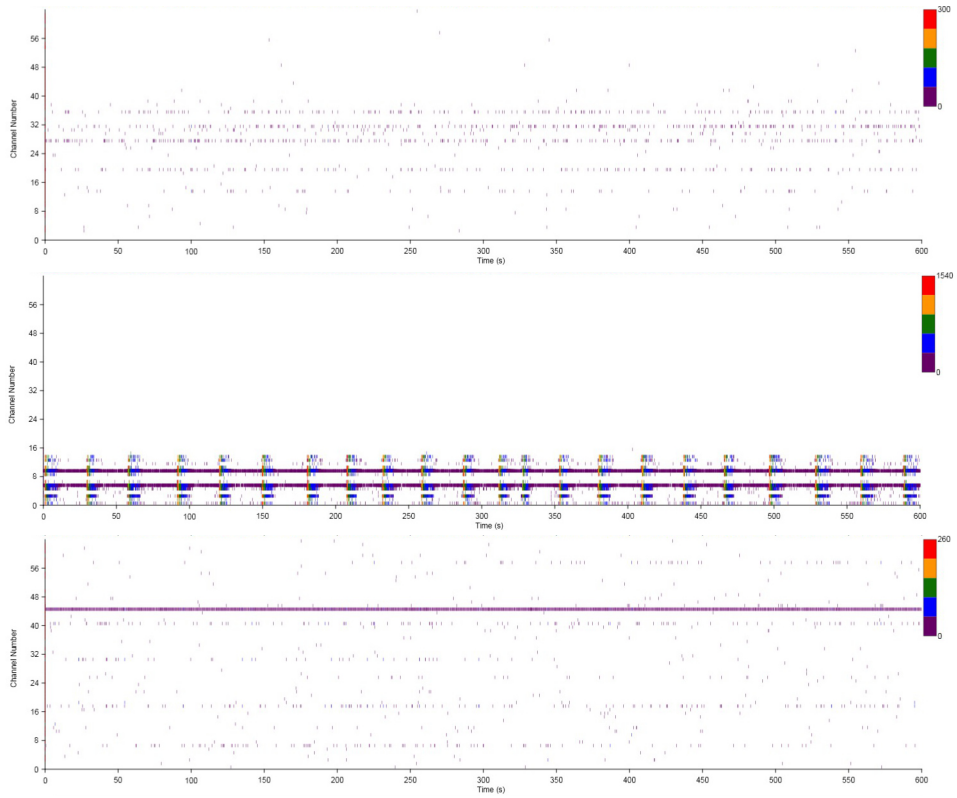

Figure 8B: *MEA data at week 11 (Rasta plots). Each group of 16 in the y axis represents the recording of one electrode, and each group of 16 in the y axis represents the recording of one well over time (x axis).* ***Top****: Dopaminergic medium;* ***Middle****: MQ Medium;* ***Bottom****: co-culture*

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