# Characterization of Early Cortical Neural Network Development in Multiwell Microelectrode Array Plates+

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## Introduction

Microelectrode array (MEA) recordings are a useful tool to study the activity of networks of interconnected neurons, both in vitro and in vivo. In vitro, neural networks on MEAs demonstrate many characteristics of intact neural networks; this includes extracellular recordings of action potentials (“spikes”) and groups of action potentials (“bursts”) simultaneously from multiple points in the network. The spontaneous activity in these networks exhibits pharmacological responsiveness and plasticity (Gross et al., 1997; Potter, 2001; Marom and Shahaf, 2002; van Pelt et al., 2005; Pasquale et al., 2008). Thus, primary cultures of neural networks on MEAs have been widely utilized to study neurophysiology, neuropharmacology and neurotoxicology (for review, see Johnstone et al., 2010). In addition, the ontogeny of network activity on MEAs has been described by numerous different laboratories (van Pelt et al., 2005; Wagenaar et al., 2006; Chiappalone et al., 2006; Illes et al., 2007; Biffi et al., 2013). Until recently, however, the throughput of MEA devices has been limited, such that it was not possible to study more than a small handful (e.g. 4-6) of networks at a time.

Recently, two manufacturers of MEA devices have introduced multi-well MEA (mwMEA) devices, which allow for recordings to be made from 12-96 wells simultaneously, with 8-64 electrodes per well. The increase in throughput offered by mwMEA devices expands the capabilities of MEA systems, allowing for drug and toxicant screening (Valdivia et al., 2014; Nicolas et al., 2014) and, in combination with genomic manipulation (MacLaren et al., 2011, Charlesworth et al. 2015b) or patient-derived inducible pluripotent stem cells (Wainger et al 2014; Woodward et al., 2014), has been used to describe how network function is affected by disease states. Finally, mwMEAs offer the ability to screen large numbers of chemicals for potential effects on developing networks (Robinette et al., 2011; Hogberg et al., 2011). Given the significant public concern over the potential role of chemicals in neurodevelopmental diseases (Landrigan and Grandjean, 2006; 2014), study of chemical effects on neural network ontogeny using mwMEAs offer a functional measure for developmental neurotoxicity hazard characterization.

However, in order for such studies to take place, the basic development of activity in networks on mwMEAs needs to be described in detail. In lower throughput MEA systems, neural network development has been demonstrated to transition from low activity at early developmental ages (e.g. the first week in vitro), to one of coordinated bursting, network spikes and synchrony at later time points (e.g. the second week in vitro and beyond). While it is expected that such properties will be retained in multi-well systems, it remains to be demonstrated, and the time-course, variability and other characteristics defined. Further, while single-well MEAs contain ~60 microelectrodes, only 12 well mwMEA plates have an equivalent number (64/well), and the extent to which network properties can be defined with fewer electrodes has not been determined.

The present studies describe the development of networks of mixed primary cortical cultures in 48 well mwMEA plates containing 16 microelectrodes/well. These cultures were prepared from newborn rat cortex and contain excitatory and inhibitory neurons as well as glia (Harrill et al., 2015). Over the first two weeks in vitro, the neurons extend axons and dendrites (Harrill et al., 2013), form synapses (Harrill et al., 2011) and, in single well MEA systems, develop spontaneous network activity (Robinette et al., 2011). The present studies characterized the ontogeny of activity of these cultures in mwMEAs by describing the firing, bursting, synchrony and network spike properties over the first 12 days in vitro (DIV).

## Methods

### Experimental Protocol

**Cell Culture.** All procedures using animals were approved by the National Health and Environmental Effects Laboratory Institutional Animal Use and Care Committee. Primary cultures were prepared from the cortex of rat pups as described previously (Valdivia et al., 2014; Harrill et al., 2015). Cells were plated (1.5 x 105 cells in a 25 µl drop of media) onto the surface of 48 well MEA plates (16 electrodes/well) that had been pre-coated with polyethylenimine (PEI) as previously described (Valdivia et al., 2014).

**MEA Recordings**. Spontaneous network activity was recorded using Axion Biosystems Maestro 768 channel amplifier and Axion Integrated Studios (AxIS) v1.9 (or later) software. The amplifier recorded from all channels simultaneously using a gain of 1200x and a sampling rate of 12.5 kHz/channel. After passing the signal through a Butterworth band-pass filter (300-5000 Hz) on-line spike detection (threshold = 8x rms noise on each channel) was done with the AxIS adaptive spike detector. On days in vitro (DIV) 5, 7, 9, and 12, plates were placed into the Maestro amplifier and allowed 5 min to equilibrate, after which 15 min of activity was recorded. All recordings were conducted at 37 °C, and since development of activity was being studied, there were no *a priori* thresholds for minimum numbers of active electrodes for inclusion of a well in the data set.

### Data

Recordings were made from 656 wells across 16 MEA plates from 15 primary cortical cultures at DIV 5, 7, 9 and 12 for a total of 64 “plate-recordings”. The majority of these plates were used on or around DIV 14 for other experiments. A recording of one plate at DIV 12 was missing from our data set, and one recording at DIV 7 was also excluded from the analysis as its very high firing rate suggested that it was an erroneous recording. This resulted in a total of 62 plate recordings, with a total of 2976 well recordings, used in our analysis. Activity was usually recorded for 15—30 minutes; only the last 15 minutes of each recording was analysed. Features related to spikes, bursts, network spikes (Eytan and Marom, 2006) and correlations (Cutts and Eglen, 2014) were extracted in the R programming environment v. 3.0 using two open source R-packages, SJEMEA and MEADQ, and compiled into a well level data set. Bursts were detected using an implementation of the MaxInterval method by Neuroexplorer (Nex Technologies, 2012), with the following threshold parameters: maximum interspike interval (ISI); 0.25 s, maximum beginning ISI; 0.1 s, minimum ISI; 0.8 s, minimum burst duration; 0.05 s and minimum number of spikes in a burst; 6. Network spikes were identified by dividing the recording period into 3ms bins and determining the number of electrodes in the well that fired at least one spike during each bin; the minimum threshold for a network spike was for spike activity to be present on at least 5 electrodes in a given time bin. Data files generated by Axis were converted into HDF5 file format (Eglen et al., 2014); HDF5 files, scripts to generate the features and related R-objects are stored in public repository (http://github.com/sje/EPAmeadev). The goal of establishing a public data set is to allow full reproducibility of our analysis and/or to allow novel analyses to be conducted.

### Developmental Analysis

Twelve features were chosen to describe the culture activity, which are summarized in Table 1. For all features, the plate value was taken as the median of all non-zero well values on the plate (zero values were ignored).

### PCA

We performed principal component analysis using the R-package FactoMineR using all wells and all 12 features. Two PCAs were performed. The first PCA was conducted using data in which a well constituted one observation, while the second PCA was conducted using data in which a plate median constituted an observation. For each PCA, the 12 dimensional feature vector was projected down onto the plane created by the first two principal component dimensions. The purpose of the projection was to visually assess the level of differentiation among the 4 ages. A scree plot was made to describe the cumulative percent of variation explained by the use of additional principal components to describe the data. The scree plots aid in quantifying the extent to which data may be well represented with fewer dimensions.

### Classification

Two classification techniques, random forests and Support Vector Machines (SVMs), were used to predict the age of each well based on the twelve features used in our analysis. In some cases, due to the low number of electrodes on a well, lack of bursting or lack of network spike activity, some feature values were missing; this was particularly evident at early DIV. For classification purposes, for those wells with no bursts, the within burst firing rate and burst duration were set to zero. Similarly, the network spike peak and duration were set to zero for all wells that exhibited no network spikes over the recording period. Any wells that had null values for the remaining features, namely correlation, CV of IBI and CV of within burst ISI, were excluded from the classification. This resulted in 370/2976 well recordings, or approximately 12.4% of the total wells, being excluded from the classification.

Initially, classification was performed on the remaining data using a random forest model and all twelve features. The relative importance of each of the features was determined based on the amount they reduced the Gini index. Next, Support Vector Machines with a radial kernel and gamma value of 1/12 were used to examine the classification accuracy obtained by using various subsets of the total twelve features.

In both types of classification, two thirds of the data were used as a training set and the remaining third used to test the classification accuracy of the model. The classification was repeated one hundred times using random choices of the training and test sets in each iteration, and the classification accuracy averaged over the one hundred repetitions.

## Results

**Developmental Profile**

On DIV 2, only rare, individual spikes were recorded (data not shown). Spontaneous activity in the neural networks arose and could be reliably recorded beginning on DIV 5 (Figure 1). Activity as assessed by most of the parameters used here increased with DIV. In particular, not only did spiking increase with time, but the organization of spiking into bursts and correlated activity across the network (Figure 1) also increased with DIV. Quantification of the changes in activity over development was achieved using a selection of twelve measures, which were used to describe activity at the level of the entire well or individual electrodes, which were then aggregated into well level values by taking the median.

*Spontaneous Firing rate*

In general, activity increased over development, with the median firing rate and within-burst firing rates both monotonically rising with increasing DIV (Figure 2).

*Bursting activity*

A clear increase in bursting activity with increasing culture age was also observed. The fraction of bursting electrodes, burst rate and percentage of spikes occurring within bursts all increased over development (Figure 3). The CV of inter-burst intervals (IBIs) and within burst inter-spike intervals (ISIs) also increased with development, indicating a decrease in the regularity of these features (Figure 3).

*Synchronous activity*

The synchrony of activity within each individual well on a plate was examined using a feature called network spikes. Network spikes were defined as short time intervals in which the number of active electrodes on the well exceeded a threshold value, and their frequency, duration and peak number of active electrodes were quantified for each plate (Figure 4). The frequency of network spikes increased with increasing developmental age. To a lesser extent, an increase in the maximum number of electrodes active during a network spike (out of a possible 16) was also observed across development.

As another measure of network synchrony, we calculated the mean of all pairwise correlation coefficients for all electrodes in a well, using the spike time tiling coefficient (Cutts and Eglen, 2014). Correlations strengthened over development, particularly at early ages (Figure 4).

**PCA**

A PCA was undertaken to visualize the level of differentiation among the four culture ages. The wells projected onto the first two PC dimensions show a stochastic organization starting from the earliest age (red, DIV 5) progressing through to the oldest age (purple, DIV 12). The progression is roughly aligned with the first PC dimension, which accounts for nearly 50% of the variation. This means that the principal mode of variation corresponds to the difference in ages of the cultures. Moreover, all factor loadings are positive on the first PC dimension, meaning that an increase in PC1 is associated with an increase in all 11 variables. Another salient aspect of the PC projection is that variation appears smaller at earlier ages. Similarly, the projection of the plate averages onto the first two PC dimensions yields a rough segregation by DIV. As in the well-level PCA, the plate average DIVs are aligned with the first PC dimension, revealing a consistent age-related characteristic to the data. A greater percentage of variability is captured by the first PC dimension (67%) as compared with the well-level PCA, related to the fact that taking averages reduces well-to-well variability. Both PCAs display sufficient visual differentiation between observations by DIV that a more thorough quantification of this separation is warranted through classification techniques.

**Classification**

Classification techniques were used to determine the degree to which the recordings could be separated into their ages using the features specified above. Firstly, a random forest model was built and used to predict the age of each well, using the twelve features from our analysis. The model was built using two-thirds of the data as a training set and its accuracy determined by using the remaining one-third of the data as a test set. When used to predict the age of each well from the four possible ages, the accuracy of the random forest model, averaged over one hundred trails, was approximately 72% (compared to the 25% accuracy that could be expected by chance).

From these random forest models, we were also able to determine the relative importance of each of the features in driving the classification (Figure 6). The two most important features were those measuring coefficients of variation, namely the CV of within burst ISI and CV of IBI. In our developmental analysis, these two features both exhibited a monotonically increasing trend with age.

Next, we used Support Vector Machines (SVMs) to quantify the degree to which recordings could be classified correctly by age when only a subset of our features was used. The SVM classifier, built using the same proportion of training and test sets specified above, had a slightly higher level of accuracy, of approximately 73%, compared to the random forest model using all twelve features. Using the ordering of feature importance found above, we were then able to analyze how prediction accuracy varied as the number of features was reduced. Table 3 shows the performance of the SVM as the number of features used in the classification was gradually reduced from twelve, in the bottom row, to just one feature, CV of within burst ISI, in row one. In general we found that the classification accuracy remained high (~70% or higher) as the number of features was reduced. However, four features (burst rate, network spike rate, CV of IBI and CV of within burst ISI) were required to maintain a prediction accuracy >65% (Table 3).

We used a similar method to examine the extent to which each pair of ages of arrays could be separated using classification techniques. In this case, rather than using all of the data in the classification, the SVM classifier was built separately on each pairwise combination of ages. The classifier was most accurate in distinguishing arrays with large differences in age, for example DIV 5 and DIV 12 arrays, for which only the top feature, CV of within burst ISI, was required to achieve almost 89% prediction accuracy (Table 4). Classification performance was poorest for pairs of arrays in which the age difference was low. For example, the prediction accuracy for distinguishing DIV 9 from DIV 12 arrays was only just above chance when using one feature. Using all features improved the ability to distinguish between closely-related ages to ~82-83%, which is well above chance (Table 4).

How many wells are needed?

In our experiments we have used all 48 wells on a plate as replicates of the same experimental condition. This is a conservative way of using the multi-well array, and an alternative, more high throughput, approach might be to use different wells for different experimental conditions. However, there is inevitably a trade off between the number of experimental conditions tested and the number of replicate recordings of conditions when assigning conditions to wells on a plate.

We therefore sought to investigate how robust our results were if fewer wells were used to form a signature of activity at a given age. Intuitively, we expected that with fewer wells we would get less reliable signatures of activity, and hence poorer classification. Rather than run experiments where fewer wells were used, we simulated the experiments by randomly removing all data for a given number of wells on each of the 16 plates, and then repeated our classification tests to see how well each age could be discriminated. Figure 7 shows that classification accuracy remained above 60% with as few as 5 wells per plate. With 16 wells (1/3 of normal), the classifier accuracy is close to the stable value. For our particular question then of discriminating the four ages, we could get reliable results using ½ (24 wells) or 1/3 (16 wells) of the data that we generated for each plate.

## Discussion

The present results describe the ontogeny of network activity in 48 well MEA plates during the first 12 days in vitro. The results demonstrated a rapid ontogeny of spiking, bursting, synchrony and network spiking activity over this period of time, which is similar to the ontogeny of activity in single well MEAs. Furthermore, these results demonstrate that by considering multiple parameters of network firing, bursting and synchrony properties, principle components analysis and classification methods can be used as reliable predictors of network age at both the plate and well levels. These results demonstrate the neural network ontogeny on mwMEAs offers, relative to single well systems, a high-throughput approach to study network development and its perturbation by drugs, chemicals and disease.

Previous studies of cortical and hippocampal network ontogeny have demonstrated that activity begins with random, single spiking activity on a single or few channels, and over a period of 2-3 weeks in vitro progresses to bursting activity which becomes more synchronous with time (van Pelt et al., 2005; Wagenaar et al., 2006; Chiappalone et al., 2006; Illes et al., 2007; Biffi et al., 2013). This is accompanied over time by the emergence of network bursts. We observed a similar ontogeny of activity in the present experiments; however, similar to previous data with this culture model (Robinette et al., 2011), the ontogeny of spiking and bursting activity occurred rapidly within the first two weeks in vitro, specifically between DIV 5 and 12 in the present study. One reason underlying the relatively more rapid ontogeny of activity in the present experiments may be the use of a culture made from early postnatal (0-24hr) rats, as opposed to other reports where embryonic (E14-E18) preparations were utilized. Another factor that may contribute to the rate of the ontogeny of activity may be the use of relatively higher culture densities, which has been shown to influence the rate and patterns of ontogeny of neural networks (Wagenaar et al., 2006; Biffi et al., 2013). In any event, the ontogeny of activity in networks cultured on mwMEA plates was qualitatively similar to that on single well plates (as expected). Furthermore, it appears that this network ontogeny can be reliably predicted by using as few as 3 wells/plate.

The use of classification techniques such as RFA and SVMs indicated that the parameters extracted from the spike trains in these experiments could be used to predict reliably the age of the culture from which data were obtained. While the models performed best when all of the parameters were used to aid classification, several parameters had greater influence on the ability to predict culture age. These included the CV of within burst ISI, the CV of the IBI, mean burst duration, network spike rate and burst rate. When these approaches were used to predict between two different ages, use of all of the parameters resulted in greater accuracy regardless of age. This indicates that using multiple parameters will provide more robust discrimination of different ages (or perhaps treatments) than relying on a single or a few parameters. The greatest accuracy was achieved when predicting between larger age differences (e.g. DIV 5 vs 12) and likely reflects the relative lack of bursts, network spikes and correlated activity in DIV 5 cultures. This is consistent with the relative lack of connectivity in DIV 5 cultures, as synaptogenesis in this model begins around DIV 6 (which is reflected by increases in electrophysiological parameters on DIV 7) and continues through DIV 12 (Harrill et al., 2011). Thus, the development of electrophysiological parameters reflecting connectivity (bursts, network spikes and correlated activity) correspond well to the development of morphological synapse formation.

The present analysis has implications for using mwMEAs for drug development or chemical developmental neurotoxicity screening. Both classification approaches used here provided higher accuracy by including more features. Traditionally, MFR has been widely utilized to describe drug- or chemical-induced alterations in network function (REFS) as it is easily extracted from the data. However, when possible, determining more features and using them collectively rather than focusing on one or a few features may provide greater sensitivity in detecting effects, as well as possibly facilitating drug or chemical “fingerprinting” approaches (Mack et al., 2014). In addition, the classification approaches used here indicate that age of a network can be reliably determined using 3-8 wells from each plate, indicating that between 6 and 16 different treatment conditions might be possible on a given 48 well mwMEA plate. We believe our study is the first to assess the important question of how to efficiently use wells on a mwMEA, suggesting that as few as 3-8 wells might suffice to form a reliable pattern of activity. However, this range should be treated with caution: as can be seen from Figure 7, there is in increasing variance with fewer wells, and more importantly, results are likely to differ depending on the size of the effect being measured. Our findings suggest that there are gross changes in activity patterns, and hence fewer wells are needed. On the other hand, where changes in activity are more subtle, we would expect more replicates to be required. Our recommendation therefore is that investigators should repeat our sampling approach (Figure 7) to investigate how reducing the number of wells per condition can affect reliability of results.

One factor that influences results from MEAs is the culture to culture variability. Although this was not specifically addressed in the present studies (typically only one plate was available from a given culture), evidence from another data set wherein three plates each were analyzed from several different cultures indicated that culture to culture variability is much greater than plate to plate variability (unpublished data). Thus, obtaining replicate values for different treatments (e.g. concentrations of a drug or chemical) across several different wells and plates from the same culture (e.g. see supplemental material in Wallace et al., 2015) may be preferable to obtaining replicate values across several different cultures. It is likely that the use of a primary culture model does contribute to the culture to culture differences, as each culture is prepared from different animals. This may in the future be improved by the use of stem cell derived models, which should be more homogeneous.

In conclusion, we have described the development of neural networks grown in 48 well mwMEA plates and found that it is qualitatively equivalent to development of network activity in single well MEAs. Furthermore, multi-parametric evaluation of the network activity parameters provides an accurate method of classifying networks by different ages. Together, these results indicate that neural networks cultured on mwMEAs will be a useful tool to study the ontogeny of network activity as well as the potential for drugs, chemicals and diseases to disrupt that activity.

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