Effects of Electrical Fields on Neuroblastoma (N2A) Cell Differentiation: Preliminary Results

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Abstract: This work describes Electrical Stimulations (ES) assays on stem cells. The neuroblastoma (N2A) cell linage was submitted to several electrical fields to enable and enhance its differentiation toward neurons. Both Direct Current (DC) and Alternated Current (AC) time dependent electric field protocols were applied to N2A cell culture under differentiation conditions, obtaining different responses. Control and electrically excited samples' number of differentiated cells and neurite lengths were measure after differentiation. Results showed that DC fields have a strong influence on N2A differentiation since the percentage of differentiated cells and the neurites lengths were the highest. In addition, a significant alignment of neurites measured with the applied electrical field has been detected, which demonstrates the high sensitivity of differentiation processes to electrical field polarity.

1 INTRODUCTION

In the most advanced organisms, each cell has a specific function, being its morphology and physiology adapted for greater efficiency carrying out that function. This process is known as differentiation. Cellular differentiation, therefore, is the process by which a cell pauses its division process and changes its function and phenotype; that is, it expresses parts of its DNA that were previously suppressed and vice versa. In our specific case, N2A cells differentiate into neurons. Undifferentiated N2A cells are small and round; when differentiation begins, the cytoplasm extends in one or more directions, forming threads called neurites, also the cell flattens (Echalier, 2018).

N2A can be cultivated in suspension or with substrate. Differentiation occurs either way; although, in suspension cultures, the morphology remains round and the number of differentiated cells decreases; these cells, once transferred to a culture with substrate, reach a normal degree of differentiation and a flat morphology. Cells cultured with substrate differentiate with a normal rate and flat morphology (Figure 1) (Ross, 1975).

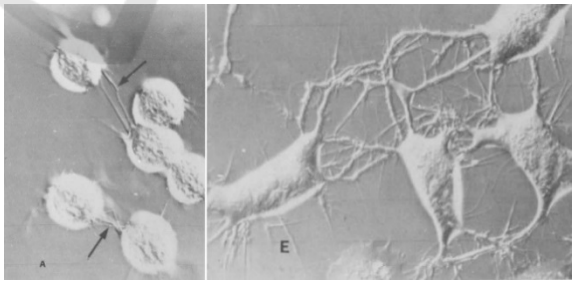

Figure 1: N2A cells in the process of differentiation: Suspension culture (left) and substrate culture (right) (Ross, 1975).

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An Electric Field (EF) plays, under the adequate electrostimulation (ES) protocol, a relevant role in the reorganization of the cell cytoskeleton (McCaig, 2005) (Villanueva, 2019). Normally, cytoskeletal reorganization is mediated, inter alia, by extracellular signaling kinase dependent (ERK) pathways. A signaling pathway is a common process at the cellular level. It starts from a chemical reactant that initiates a series of reactions within the cell; this ultimately produces a result. Signaling pathways are the primary method most cells use to transmit and translate chemical signals. The ERK pathway produces cytoskeletal reorganization and, ultimately, cell differentiation. One of the steps in this signaling pathway is dependent on cyclic adenosine monophosphate (cAMP) (Liu, 2001). Several studies have demonstrated the efficacy of cAMP (Chang, 1976), or of factors that increase cAMP activity (Chatterjee, 1992), (Tremblay, 2009), inducing cell differentiation in N2A, as well as the efficacy of β - Hydroxy-β-methylbutyrate (HMB). HMB, in turn, activates the ERK signaling cascade (Salto, 2015). There are also authors that claim that cyclic AMP acts similarly, when it is activated by an electric field and it is activated by chemical factors (Pullar, 2005), when it comes to migrating keratinocytes. This could prove that the application of an electric field produces the activation of cAMP. With this information, it is possible to link electrostimulation and cell differentiation. There could be a relationship between the application of electrostimulation and the ERK signaling cascade; possibly involving the activation of cyclic AMP. Therefore, an electric field could use the ERK signaling pathway to reorganize the cytoskeleton and ultimately lead to cell differentiation, by activating cAMP.

The main objective of this work is to promote the differentiation and growth of neurites in mouse N2A cells, applying electric fields during the differentiation process. To achieve that, two experiments will be carried out with different electrostimulation protocols, keeping the same cell incubation protocol. Experiments are planned to compare the efficacy of both excitation protocols enabling the differentiation of N2A cells into neurons. The proposed electrostimulation system main blocks are shown in Figure 2. The functional blocks are: the electrodes for cell culture; the circuits for electrical stimulation, generating the proposed signals, and the electrical system encapsulation for insulation inside the incubator. Two electrical signals (protocols) are applied: DC, with several values, and squared signals at the same frequency and duty cycles, modifying its amplitude.

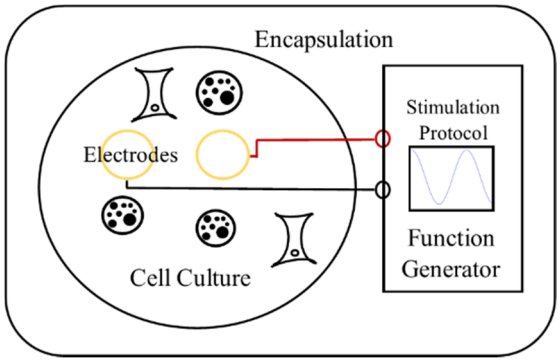

Figure 2: Main blocks in proposed electrostimulation system.

2 MATERIALS AND METHODS

This section will describe the experimental procedure followed on electrostimulation assays with N2A.

2.1 N2A Cell Culture

Initially, 25,000 cells were seeded for the first experiment (DC) and 12,500 cells for the second (AC), both in 600ml, located on each of the 8 wells of the plate, that will be explained in the next section. The DC culture grew for 72 hours and the AC for 96 hours. For sample preparation, DNEM (Dulbecco's Modified Eagle Medium) was added initially. Subsequently, they were placed in an incubator at 37ºC with 5% CO2 for 24 hours. This medium was then removed and serum-free medium (Opti-MEM, Reduced Serum Medium) was added for the cells to differentiate, and returned to the incubator for 24 hours more. After that time, electrostimulation was carried out for 6 hours. Once 18 hours more of incubation had passed, the experiment was finished (Figure 3) and the results obtained were evaluated.

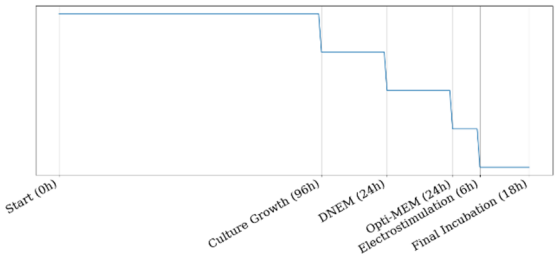

Figure 3: AC incubation protocol.

2.2 Electrodes

The choice of electrodes is important. The results will depend on the material from which they are made of,

their geometry, and layout. In many studies, the culture is made in Petri dishes and, therefore, there is total freedom in the choice of electrodes and their configuration. The best electrode arrangement is calculated then, as well as some way to place them in that arrangement. In our case, the culture took place in wells on a plate with electrodes, so all these drawbacks were eliminated. The chosen plate is specifically designed for Electrical Cell Impedance Sensing (ECIS) techniques, being applicable directly to electrostimulation assays. As advantages: it can be used with large numbers of cells, it reduces impedance fluctuations, its electrodes are uniformly arranged in space, and all wells hold the same volume. The cells were cultured in wells located in a commercial plate from Applied Biophysics (Applied Biophysics, 2020) using the model 8W10E+ from the ECIS Cultureware line shown in Figure 4.

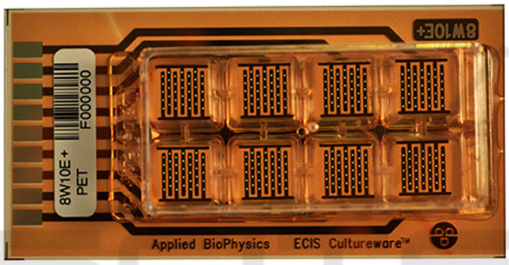

Figure 4: AB Electrodes (Applied Biophysics, 2020).

Each of the eight wells has two sets of 20 circular electrodes with a diameter of 250µm. The electrodes are arranged in an interlocking fingers (interdigit) configuration, where sets of five electrodes face each other; one set with the signal and one with the reference, as it is illustrated in Figure 5.

The electrostimulation signal, therefore, will be applied from the working to the reference electrodes and vice versa. It was necessary to calculate the separation between both electrodes to apply the electric field with the correct amplitude, in terms of volts by centimeter [V/cm]. For this end, the distance tool within Matlab image tools, was employed. Knowing that the diameter of the electrode is 250 µm, we could extrapolate the distance between the electrodes. We calculate the distance between electrodes, in pixels, as the average of the measurements taken, and we applied the conversion factor calculated previously. The result is 0.947mm, approximately. With this data, we were able to transform the amplitudes defined in V/cm to specific voltages for our experimental setup.

Figure 5: Electrode configuration inside a well. There are 20 gold electrodes in parallel (working electrode) vs 20 gold electrodes in parallel (reference electrode). Each circular electrode has a 250µm diameter. Model 8W10E+.

2.3 Stimulation Circuits. ES Signals

2.3.1 DC ES Signals

In the first experiment, four direct current signals were used, one of them being ground. The characteristics of the signals were chosen according to the bibliography. The main work contributions to DC electrostimulation of N2A cells and similar are in Table 1. The chosen amplitude values are consistent with the values that other authors have used in their studies, and specifically with this cell type.

Table 1: Summary of the voltage amplitudes reported for DC electrostimulation.

Cell line	Amplitude (V/cm)	Ref.
N2A	1.1-10	(Jain, 2013)
SH-SY5Y	1.5	(Xiong, 2015)
Xenopus Neurons	0.1 - 10	(Jaffe, 1977)

Considering the values in Table I, and the distance between electrodes of 1mm, the V/cm values are calculated for our electrode setup. The final DC amplitudes in our assay are listed in Table 2.

Table 2: Amplitude selected for the DC assay.

Amplitude	1	2	3	4 (GND)
(mV)	125	250	500	0
(V/cm)	1.25	2.5	5	0

For the electrostimulation circuit, an inverter amplifier was used, built with an operational amplifier and two resistances, with its gain defined by the resistance's ratio.

2.3.2 AC ES Signals

In the case of alternating current, more parameters had to be decided, not only the amplitude, but also the waveform, frequency and pulse width (Table 3). Three different signals were applied. To make comparisons between setups and to facilitate the assembly of the circuit, it was decided to apply the same signal with three different amplitudes, as in the case of direct current. Ours, as well as other possible proposals are listed in Table 3. The squared signal proposed in this work is illustrated in Figure 6.

Table 3: ES parameters for AC assays.

Signal	Freq [Hz]	Pulse width	Amplitude	Ref.
Squa. Bipha.	100	2.5ms pos 2.5ms neg	125,250, 500 mV/mm	This work
Squa. Bipha	*n.a.*	200µs–1ms	100mV-2V	(Braeken, 2009)
Squa. Bipha	100	50-200µs	4 - 32µA/cm	(Chang, 2011)
Sin	1,10,50	----	1V/cm	(Lim, 2013)
Squa.	100	5ms	300mV/mm	(Chang, 2016)
Squa.	1	2ms	5V	(Xiong, 2015)
Squa.	1,3,5	0.25–10ms	1 – 6V/cm	(Tandon, 2011)

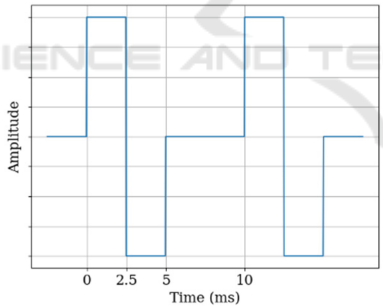

Figure 6: Proposed waveform for the AC signal. Three amplitudes are considered. Frequency is 100Hz, and the selected amplitudes are 100mV, 200mV and 425mV.

The timer circuit generates square pulses of frequency 100Hz and pulse width 2.5ms; in addition to a circuit that varied its amplitude. It was decided to use assemblies based on the 555 integrated circuit (IC) for the timer and differential amplifiers based opamps for the amplitude variation. The timing circuit needed a square pulse generator and two circuits to regulate the width of the pulse, one for the positive pulse and one for the negative pulse; both pulses had to happen one immediately after the other. With two IC 555s in monostable mode we would be able to do that. Later we would use a differential amplifier to invert the negative pulse and combine it with the positive one, in addition to regulating the amplitude of both. In this from, we would have the complete circuit to generate one of the three AC signals. Each, DC and AC signal is applied to two wells. Figure 7 displays the plate layout of the DC signals applied. AC is similar.

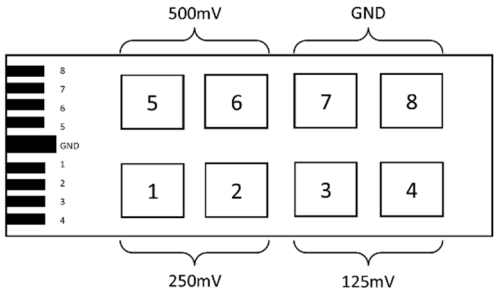

Figure 7: Proposed DC signals layout. Each DC signal is applied to two wells. Wells 7 and 8 are the controls.

2.4 System Encapsulation

The whole electrostimulation system, circuits plus electrodes, was put inside an incubator during experiments. Cells in the plate were set over the cage, while the circuits were placed inside it, to insulate them from the incubators conditions (Figure 8).

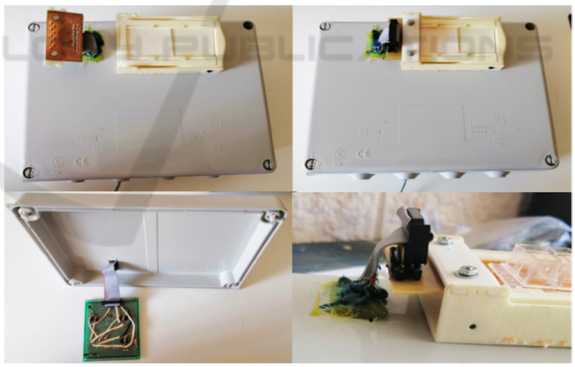

Figure 8: Cage system: electrode wells are put over the cage and the electrostimulation circuits inside.

2.5 Cell Measurements

Several measurements were performed, to evaluate the performance assay.

- Number of cells
- Number of differentiated cells
- Number of neurites
- Neurite lengths
- Neurite polarization

3 EXPERIMENTAL RESULTS

The experimentation process consists of several parts: the first is the culture of N2A cells, then electrostimulation is carried out and, finally, photographs are taken under the microscope of each electrode. This procedure lasted three days, during which the direct current and alternating current experiments were carried out in an overlapping manner. The cultures had been prepared previously, according to the procedure detailed in the Material and Methods section.

After the stimulation process, an optical microscope and a Leica family camera were used to take the snapshots, communicating to a computer using the LAS EZ software (Leica, 2020). Using this procedure, we were able to take pictures of each electrode, whilst adjusting parameters such as exposition, gain, gamma, etc. to highlight neurites on differentiated cells. Only images that provided information were taken, that is, electrodes with multiple layers of cells or damaged electrodes would not be photographed. The photographs were stored in folders according to their well.

From the pictures taken, the several paramezters proposed to characterize the experiment were measure: number of cells, number of differentiated cells, number of neurites, length of neurites and neurite polarization. To distinguish between differentiated and undifferentiated cells, a criterion based on morphology was followed: cells with extensions (neurites) are differentiated cells (Figure 9). On the other hand, it was necessary to distinguish between living and dead cells. Dead cells are normally found in “spongy”-looking aggregates (Figure 10).

With clear criteria, the cells were counted first. A Matlab script was programmed for this task (Figure 11). The script opens the images found in the folder (corresponding to a well) one by one, that is, it opens one and, when given the order, closes it and opens the next one. Once an image is opened, the view is zoomed in, in order to visualize the electrode better; click where a cell is to count it, and when all have been counted, proceed with the differentiated cells. After that, the image will be closed and the next one will be opened. Once finished, the script will generate a .csv file with the number of total cells in each image, another with the differentiated cells and another with the coordinates of the points where the differentiated cells are (Figure 12).

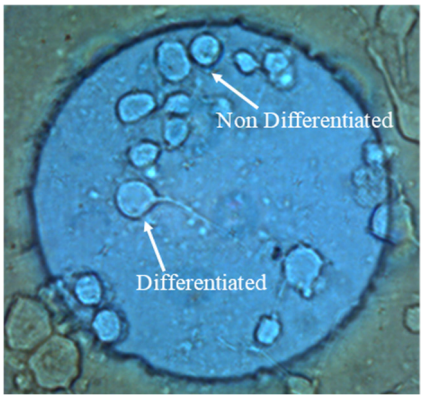

Figure 9: Example: differentiated and non-differentiated cell.

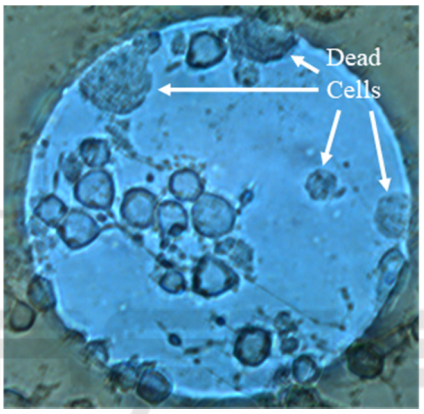

Figure 10: Example: dead cells.

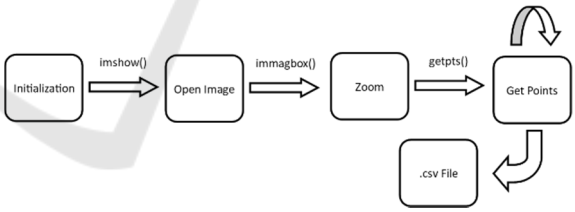

Figure 11: Cell counting Matlab script block diagram.

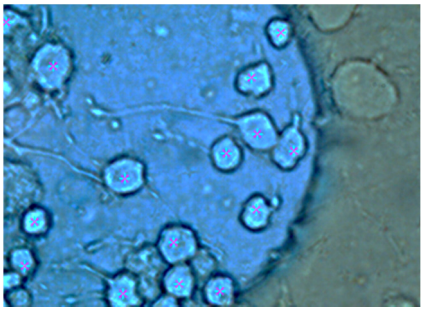

Figure 12: Matlab display when cells are being counted.

The next step was to count and measure the neurites of the differentiated cells. For this, another script has been developed in Matlab (Figure 13). This script opens the images in a similar way to the previous one, in this case the coordinates of the points calculated in the last step are read in order to mark the differentiated cells with a red cross. In turn, a ruler with two moving points is shown. The ruler must be placed at the beginning and end of the neurite and when given the order, the length of the neurite, its starting point and its end are recorded. When the last neurite of an image is measured, the order is given and the image changes to the next. Subsequently, two .csv files are generated, one with the distances of the neurites per electrode and the other with the starting and ending points of the neurites (Figure 14).

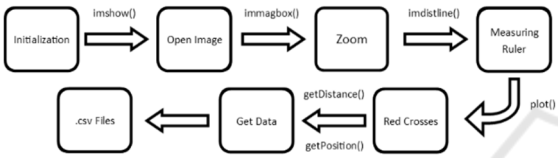

Figure 13: Neurite length measuring Matlab script block diagram.

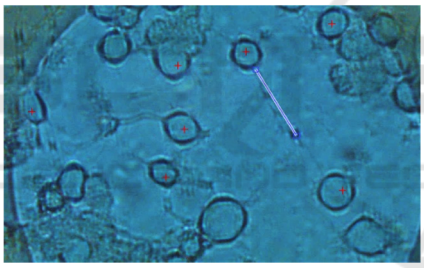

Figure 14: Example measuring the neurite length with Matlab.

As additional information, the orientation of the neurites has also been obtained, that is, the angle they form in the image, respect a given coordinate origin. This was possible thanks to the nature of the information extraction process from the images. By knowing the start and end points of the neurite (Figure 15), it was possible to obtain the angle it forms, using the inverse of the tangent; as shown in Equation (1). This approximation, although not perfect, allows us to recognize some type of pattern in the angles of the neurites, if it exists.

$$\theta = \tan^{-1}\frac{y}{x} \quad (1)$$

Where y = (y1-y2), x = (x1-x2) and Θ is the angle that the neurite forms (Figure 15).

An extensive analysis was performed to obtain the expected measurements from the experiment. In the following are summarized some of the more relevant, illustrating the sensitivity of N2A cells to DC and AC electric fields. Figures 16 and 17 show the mean value of the number of differentiated cells in DC and AC conditions, for the several values of amplitudes applied.

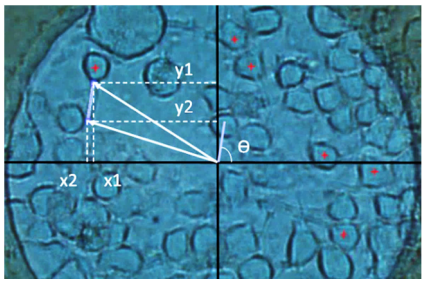

Figure 15: Parameters used in the calculus of the neurite angle. The center of the electrode is taken as reference axis.

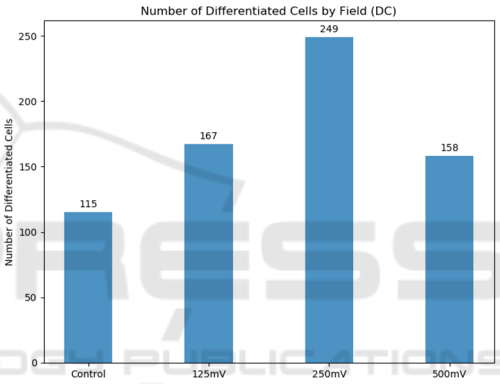

Figure 16: Number of differentiate cells vs DC level.

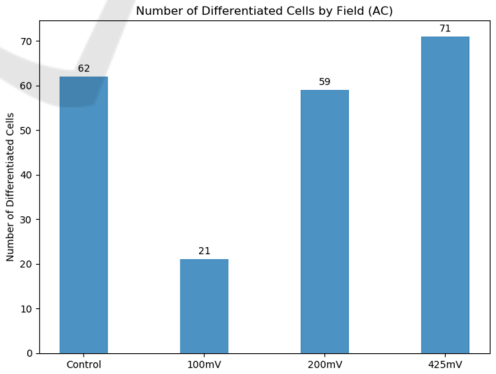

Figure 17: Number of differentiate cells vs AC level.

The percentage of differentiate cell, with respect to the total number of counted cells is displayed in Figures 18 and 19, for DC and AC conditions respectively. The efficiency on differentiation seems to be higher for DC electrical fields, being highest at 125mV (1.25V/cm).

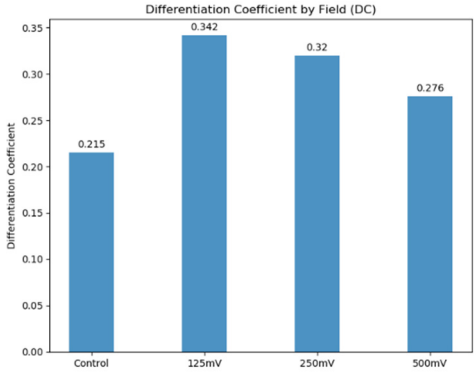

Figure 18: Differentiation coefficient for DC.

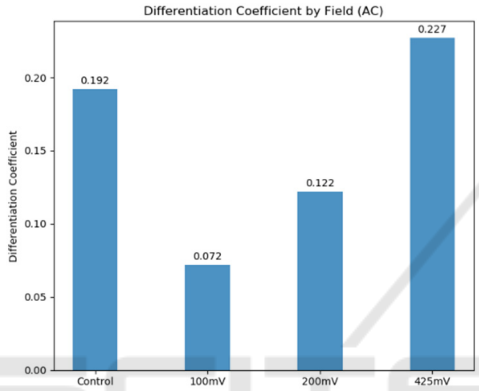

Figure 19: Differentiation coefficient for AC.

Finally, the phase angle tested over the photographs for the two assays (DC and AC) are displayed on Figures 20 and 21, together with their respective controls. In both cases (DC and AC), it can be appreciated a high polarization around (25º- 40º) and (130º-150º) intervals, validating the fact that one of the neurite answers to electrical field is its biasing along the applied ES electric field (Patel, 1982).

The presented results show how N2A cells can be electro-stimulated using lateral electric fields defined from the bottom electrodes, and how their neurites were biased along the direction defined by the electric fields applied.

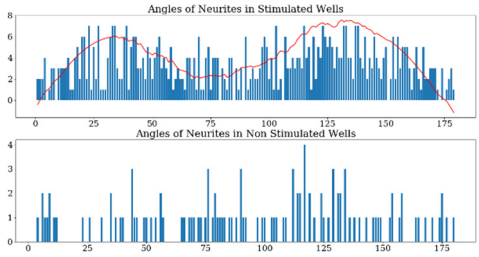

Figure 20: Neurites polarization (DC): Number of neurites measure for each angle between 0º and 180º. The red line represents an approximated fitting curve.

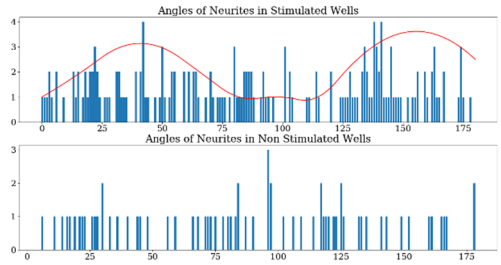

Figure 21: Neurites polarization (AC): Number of neurites measure for each angle between 0º and 180º. The red line represents an approximated fitting curve.

4 CONCLUSIONS

An experimental procedure and the results of electrostimulation assays on neuroblastoma cells, in the process of differentiation towards neurons, have been presented. Two types of signals were utilized on the experiments: the first one consisting of a direct current voltage signal and the second one a biphasic square voltage signals at 100 Hz, both with three different amplitudes. The results obtained demonstrate that, for the selected amplitude values, the differentiation process is more sensitive to DC than to AC signals, with similar amplitudes. This could be due to the biphasic nature of the AC signal applied. A biphasic signal polarizes cells in the two directions of the electrical field, whilst a DC signal only polarizes towards the cathode. Another factor to keep in mind is the rms value of the AC, this being, approximately, 0.71 times the amplitude of the signal. Therefore, the AC signals applied have less effective amplitude than the DC ones. This could also explain the tendency seen in Figure 19, where the coefficient increases as the amplitude does. The optimum lateral ES field values, 100mV/mm, is coincident with the reported by other authors (Jain, 2013), using vertical electrical fields over its setup. This result supported the validity of the assays. A remarkable alignment of the neurites was observed on the maximum electric field directions expected (30º – 150º), which means a polarization response of N2A cells to external stimulus, as stated in (Patel, 1982). More in-depth experiments must be done in AC ES, for wider frequency ranges and duty cycles, to fully characterize ES signals effects on N2A differentiation. In addition, higher/smaller ES periods of 6h should be tested, for the same reason. Finally, as future work, it is programmed to incorporated some biomarkers, as cAMP, in parallel with electrostimulation, for biological validation of the assays.

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