# Oxygen-controlled automated neural differentiation of mouse embryonic stem cells

**Authors, Affiliations and Correspondence:**

Mondragon-Teran P[‡]., Tostoes R., Mason C., Lye G.J., Veraitch F.S.*

The Advanced Centre for Biochemical Engineering, Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE UK

[‡]Present address: Biomedical Research Division, Centro Medico Nacional "20 de Noviembre" – ISSSTE. San Lorenzo 502, Del Valle, Benito Juarez, México City, 03229 México

*Corresponding author

email: f.veraitch@ucl.ac.uk

tel: +44 (0) 20 7679 2648

fax: +44 (0) 20 7209 0703

**Key words:**

Embryonic stem cells, neural differentiation, oxygen tension, automation

**Summary**

Automation and oxygen tension control are two tools, which provide significant improvements to the reproducibility and efficiency of stem cell productions processes. **Aim:** The aim of this study was to establish a novel automation platform capable of controlling oxygen tension during both the cell culture and liquid handling steps of neural differentiation processes. **Materials and Methods:** We built a bespoke automation platform, which enclosed a liquid handling platform in sterile, oxygen-controlled environment. An airtight connection was used to transfer cell culture plates to and from an automated oxygen-controlled incubator. **Results:** Our results demonstrate that our system yielded comparable cells numbers, viabilities, metabolism profiles and differentiation efficiencies when compared with traditional manual processes. Interestingly, eliminating exposure to ambient conditions during the liquid handling stage resulted significant improvements in the yield of MAP2 positive neural cells indicating that this level of control can improve differentiation processes. **Conclusions:** This article describes, for the first time, an automation platform capable of maintaining oxygen tension control during both the cell culture and liquid handling stages of a two dimensional differentiation process.

## 1 Introduction

Embryonic stem cells (ESC) are currently being evaluated for potential application in a number of diverse areas including regenerative medicine [1], drug discovery and development [2-4] and as routes for delivery of gene therapies [5]. The high level of interest is a consequence of the ability of ESCs to self renew indefinitely and to differentiate into most adult cell types [6-10]. The production of differentiated cell types from ESC will be reliant upon robust, reproducible and high yield cell culture processes [1, 11,12]. Automated bioreactors have historically allowed the control of mammalian suspension cell culture resulting in repeatable and controlled bioprocesses [13,14]. Although a number of studies have used microcarrier technologies to differentiate ESC in suspension bioreactors [15-22] the vast majority of current differentiation protocols are based on 2D cell cultures.

A number of reports have used commercially available automation platforms for the maintenance of stem cells under 2D culture conditions [12, 23-28]. By eliminating reliance upon highly skilled operators these systems can enhance the reproducibility of stem cell bioprocesses. However, these automated platforms do not control the exposure of cells to key gaseous components, such as oxygen, during the robotic liquid handling steps of ESC culture.

Physiological oxygen levels that occur during early embryo development have been shown to maintain ESC in a pluripotent state [29-31] and to improve their directed differentiation towards cardiac [15, 20], vascular [32], neural [33-37] and retinal lineages

[38]. Studies from our laboratory revealed that the yield of neural cells from mouse ESC (mESC) can be greatly enhanced by manually operating at 2% $O_2$ [33]. However, the oxygen tension at the growth surface rose to 21% $O_2$ during medium exchanges. It took approximately 5 h for the oxygen tension at the growth surface to return to 2% $O_2$ once the cell culture dishes had been returned to a hypoxic environment. The biological repercussions of these transient shifts in oxygen remain unclear. In a separate study we also revealed that both the expansion and differentiation of mESCs were sensitive to shifts in pH and temperature which were detected when cells are removed from the incubator and processed under ambient conditions [39].

In this article, we present a novel automation platform for 2D stem cell culture capable of controlling oxygen tension during the medium exchange step. By integrating an oxygen controlled incubator into the platform we were able to maintain 2% $O_2$ during both the culture and liquid handling steps. Using this system, mESC were differentiated into neural cells whilst maintaining 37$^{o}$C, 5% $CO_2$ and 2% $O_2$ throughout the entire 8 day differentiation protocol.

## 2 Methods and Materials

### 2. 1 Undifferentiated cell culture

E14Tg2a mESC, kindly donated by Stem Cell Sciences Ltd (Cambridge, UK), were maintained in culture at 37°C and 5% $CO_2$ under sterile conditions in a Heraeus Hera-Cell 150 incubator (Jencons-PLS, West Sussex, UK). Cells were grown in 5 ml of

culture media on Iwaki T25 tissue culture treated flasks (SLS, Nottingham, UK) coated with 0.1% gelatin (Sigma, Poole, UK). The culture media was composed of: 450 ml of Glasgow minimal essential media (GMEM) supplemented with 500 μl of 0.1 M 2-β-mercaptoethanol (both Sigma), 50 ml foetal bovine serum, 5 ml non-essential amino acids, 5 ml of 200 mM L-glutamine and 5 ml of 100 mM sodium pyruvate (all Invitrogen, Paisley, UK). After sterilization by filtration the media was supplemented with 0.5 ml of $1x10^6$ units.$ml^{-1}$ leukemia inhibitory factor (LIF, Millipore, Hertfordshire, UK). Cells were passaged after 2 days of culture by removing spent media followed by a gentle wash with Dulbecco's phosphate buffered saline without $Ca^{2+}$/$Mg^{2+}$ (DPBS, Sigma). Cells were detached with a solution of 0.025% trypsin, 0.04% ethylenediaminetetraacetic acid (EDTA, all Invitrogen) and 0.9% chick serum (Sigma) dissolved in DPBS. After 3 minutes of tripsinization at 37°C, 3 ml of fresh growth media were added to quench the trypsin. Cells were centrifuged for 3 minutes at 300 g, resuspended as a single cell suspension in growth media and re-inoculated into a new T25 flask pre-coated with gelatin. The E14Tg2a mESC used in all experiments were between passage 15 and 22. Oct3/4 and Nanog pluripotency marker expression were used to assess the quality of the cells by immunocytochemistry (ICC) (Supplementary Figure 1).

**2.2 Neural Differentiation Protocol**

Monolayer neural differentiation was based on the method reported by Ying *et al.*, [40]. Undifferentiated stem cells were harvested after 2 days of culture by tripsinization. Trypsin was quenched with fresh culture media without LIF. The resulting cell

suspension was centrifuged for 3 min at 300g, resuspended in NDIFF-RHBA differentiation culture media (Stem Cells Sciences). Cells were plated in 2 ml of medium at a density of 2 x $10^4$ cells.$cm^{-2}$ in Iwaki tissue culture treated six-well plates (SLS, Nottingham, UK) pre-coated with 0.1% gelatin (Sigma). Each well was replenished with 2 ml of NDIFF-RHBA media every 2 days in a laminar flow hood operating under normal atmospheric conditions over a total 8-day culture period. Manually seeded six-well plates were also transferred to hypoxic chambers and the automated incubators for processing at 2% $O_2$. When required cells were trypsinized and gently resuspended with a Pasteur glass pipette to recover a single cell suspension for further analysis.

**2.3 Description of the automation platform**

A Tecan Freedom EVO 100 liquid handling platform (Tecan, Mannedorf, Switzerland) was used to automate medium exchanges during the neural differentiation protocol. The platform was contained within an enclosed class II biological safety cabinet (Walker Safety Cabinets, Derbyshire, UK) (Figure 1.a). This system has the capability to control temperature, $O_2$ and $CO_2$ during liquid handling stages. Airtight connections were used to integrate a microplate centrifuge (Hettich Rotanta 46 RSC, Bach, Switzerland) and an automated Thermo Cytomat C450S incubator (Thermo-Fisher, Basingstoke, UK). The incubator was equipped with a 32 plate position rack, temperature, $O_2$, $CO_2$ and humidity control and a robotic arm for the selection and transport of culture plates to the liquid handling platform. The liquid handling platform was composed of the following devices: liquid handling arm with 4 channels and

integrated level liquid detection (LID), robotic manipulator arm (RoMa), robotic transport arm (Te-Link), tilting plate holder and a media reservoir station consisting of 2 x 500 ml and 5 x 100 ml wells designed to accept sterile disposable medium containers (Tecan) (Figure 1.b). The liquid handling arm was equipped with 1 mL syringes, which were used with 1 ml disposable tips (Tecan). Disposable tips and fresh medium were transferred onto the robot deck using an airlock which was located to the left of the platform (Figure 1a). All the robotic arms and tilting plate holder were controlled through Freedom EVOware 2 plus software (Tecan).

**2.4 Automated processing parameters**

In order to avoid the exposure of differentiating cells to ambient conditions during the liquid handling steps the environment within the class II safety cabinet was maintained at a set point of 37ºC, 5% $CO_2$ and 2% $O_2$. Every 48 hours plates were transported onto the liquid handling platform by the incubator's robotic arm with a maximum speed of 500 $mm.s^{-1}$ and placed in the same position through the use of a sensor with an accuracy of ± 0.5 mm. Plates were picked up from the robot arm by the Te-link transporter arm, using a 10-15 N gripper force, and transferred to the tilting plate holder. The tilting plate holder moved at a speed of 100 $mm \cdot s^{-1}$ until it reached a position of 30º relative to the robotic deck. At this position, all the spent medium was removed by aspiration and replaced by fresh medium that had been allowed to equilibrate for at least 5 hours on the robot deck. The speed of aspiration to discard and dispense media during culture media renew was 150 ± 7.5 $\mu l \cdot s^{-1}$ under a pipetting working pressure of 1 mPa.

Tips were positioned at the corner of the plate, 5 mm from the culture surface of the tilted plate with a separation of 38 mm between tips, this enabled simultaneous multiple well processing. Figure 2 shows an overview of all the stages involved in automated processing.

**2.5 Oxygen tension measurements**

The oxygen tension was measured using the Presens Oxy-4 system in conjunction with Sp-pst3-NAU-D5-NOP oxygen sensor spots (both Presens, Regensburg, Germany). Presens' detection principle is based on the quenching of fluorescence caused by collision between molecular oxygen and oxygen sensitive fluorescent dye molecules. The fluorescent dye molecules are immobilized in the form of a spot and coated with an optical insulation to avoid interference from intrinsic fluorescence. An oxygen sensor spot was used to monitor the oxygen tension at the growth surface of 6 well plates. The spots were placed at the base of individual wells before being submerged with 2 ml of NDIFF-RHBA differentiation media. The 6 well plates were placed in the automated incubator and the liquid handling platform under operation at 2% $O_2$, 5% $CO_2$ and 37$^{o}$C. The Oxy-4 system and sensor spots were calibrated before each experiment in accordance with the manufacturer’s instructions which can be found at www.presens.de.

**2.6 Oxygen tension control for manual processing**

Oxygen control was achieved by the use of an in-house developed hypoxia chamber [33]. Hypoxia chambers were able to hold up to 3 x 6-well plates and a 10 cm diameter Petri dish (SLS) containing 5 ml of sterile $H_2O$ for humidification. Once the 6 well plates and Petri dish were placed inside the hypoxia chamber, a removable cover was attached, tightly sealed and purged with the premixed gas containing 2% $O_2$ supplemented with 5% $CO_2$ and balanced with 93% $N_2$ (BOC, London, UK). Hypoxia chambers had a filter-inlet and outlet of gas to allow sterile gassing of the system performed for 5 min at a flow rate of 7.5 $l.min^{-1}$, after which the tubing was sealed using two screw clamps. Gassed and sealed hypoxia chambers were placed in incubators to control temperature at 37°C during the culture period.

**2.7 Cell number and viability**

Cell number and viability was measured every 48 hours using Guava ViaCount reagent (Millipore, California, US) according to manufacturer's instructions. Samples were analyzed on a Guava EasyCyte 96-well flow cytometer using Cytosoft 3.6.1 – Express Plus software to detect and calculate cell number and viability.

**2.8 Immunocytochemistry**

Immunocytochemistry (ICC) analysis was performed at the end of the 8 day differentiation protocol. NDIFF-RHBA spent media was discarded and samples were gently washed with DPBS for 5 minutes followed by fixation with 4% para-formaldehyde

(Sigma) for 20 minutes at room temperature. After the fixation step, cells were gently washed with DPBS and incubated during 30 minutes with permeation solution composed of 0.25% Triton X100 in DPBS without $Ca^{2+}$/$Mg^{2+}$ (Sigma). The permeation solution was subsequently discarded and cells were incubated at room temperature for 60 minutes with primary antibody. Primary antibodies anti-MAP2 (1:500), anti-βIII tubulin (1:400, both Sigma), anti-Nestin (1:300, Millipore), anti-Oct3/4 (1:200, Santa Cruz) and anti Nanog (1:300, Abcam) were diluted in blocking solution composed of 0.25% Triton X100 and 2% goat serum in DPBS without $Ca^{2+}$/$Mg^{2+}$ (Sigma). Mouse IgG2b isotype control for βIII tubulin and mouse IgG1 isotype control (both Sigma) for Nestin and MAP2 were used at the same dilution as the corresponding primary antibodies. Cells were washed twice for 5 minutes with DPBS to discard the excess of primary or isotype antibodies. Anti-mouse IgG (Fc Specific) FITC conjugate (Sigma) diluted 1:400 in blocking solution was used as secondary antibody by incubation for 60 minutes at room temperature followed by 2 wash steps with DPBS for 5 minutes. Samples were stained with a 4',6-diamidino-2-phenylindole (DAPI) solution at a concentration of $1\mu g \cdot ml^{-1}$ for 5 minutes for nuclei identification. An inverted fluorescent Nikon Eclipse TE2000-U microscope was used for image recording. High resolution images of MAP2 and βIII tubulin staining are shown in Supplementary Figure 2 confirming the correct localization of each antibody. The number and diameter of βIII tubulin positive neural rosettes from 30 random fields of view was evaluated in triplicate wells. Neural rosettes were manually counted for each field of view. The diameter was obtained using image-processing software (Image J, NIH, USA) by measuring the maximum and minimum diameter of each neural rosette before calculating the mean value.

## 2. 9 Flow cytometry analysis of marker expression

At the end of differentiation protocol (8 days) cells were stained for nestin, βIII tubulin and MAP2 using the ICC method previously described without DAPI staining. Immunostained samples were trypsinised for 5-10 minutes at 37°C. In order to obtain a single cell suspension for flow cytometry analysis, cells were gently re-suspended using a Pasteur glass pipette. Samples were analyzed on a Guava EasyCyte 96-well flow cytometer using Cytosoft 3.6.1 – Express Plus software. Cells immunostained with isotype controls were used to gate the negative population allowing the detection of 99 % population expressing each of the neural markers.

## 2.10 Metabolite analysis

Nova Bioprofile 400 instrument (Nova Biomedical, Flintshire, UK) was used to determine levels of glucose, lactate and ammonium in the spent media. These measurements were used to calculate molar concentrations at specific time points as well as to calculate specific rates of consumption and production. Instrument calibration according to manufacturer's instructions was always performed prior to sample measurements.

## 2.11 Calculation methods

2.11.1 Specific growth rate (μ) and doubling time ($t_d$)

The specific growth rate (μ) values were calculated during the exponential phase of the culture (from day 4 to day 8 post seeding) using equation 1:

$$\mu = ln\left(\frac{X_i}{X_{i-1}}\right) \times \frac{1}{\Delta t} \quad (1)$$

Where μ corresponds to the specific growth rate value at any given time interval $\Delta t$ (in days, between sampling points *i-1* and *i*), and *X* (cells) is the value of viable cells at each corresponding time point. The exponential phase of each culture condition allowed the calculation of 3 specific growth rate values from which the average and standard deviation were calculated and presented in reciprocal days ($day^{-1}$).

From equation 1 the doubling ($t_d$) time was calculated as follows:

$$t_d = \frac{\ln(2)}{\mu} \quad (2)$$

2.11.2 Calculation of the specific rates of glucose consumption and lactate production (q)

The specific production and consumption rates of glucose and lactate were calculated using equation 3:

$$q = \frac{\Delta C}{\Delta t} \times \frac{1}{X_{average}} \quad (3)$$

Where $q$ (in pmol.cell$^{-1}$.day$^{-1}$) represents the specific consumption (for glucose) or production rate (for lactate), $\Delta C$ is the change in the concentration of glucose or lactate ($C_{t-1}$-$C_t$ and $C_t$-$C_{t-1}$, respectively), $\Delta t$ is the corresponding time and $X_{ave}$ is the average volumetric cell concentration between *i-1* and *i*. The specific rates are presented in pmol.cell$^{-1}$.day$^{-1}$

### 2.11.3 Calculation of the fold increase in cell density

The fold increase in cell density was calculated using equation 4

$$FI = \frac{X_{2\%O2}\, P_{2\%O2}}{X_{20\%O2}\, P_{20\%O2}} \quad (4)$$

Where:

$X_{2\%O2}$ is the viable cell density at 2%$O_2$ (either manual or automated).

$P_{2\%O2}$ is the percentage of cells expressing each marker (nestin, βIII tubulin or MAP2) at 2% $O_2$ (either manual or automated).

$X_{20\%O2}$ is the viable cell density at 20% $O_2$ condition

$P_{20\%O2}$ is the percentage of cells expressing each marker (nestin, βIII tubulin or MAP2) at 20% $O_2$ condition.

### 2.13 Statistical analysis

Three replicates were performed for all cell culture experiments. Error bars represent one standard deviation above and below the mean. Significant differences between data points were calculated using a two-tailed, paired, student t-test. P values of less than 0.05 were deemed to be significant.

## 3. Results

In order to investigate the impact of environment-control during cell culture liquid handling steps, we compared the neural differentiation of mESC under three conditions. Cell culture plates were either cultured in an incubator under atmospheric conditions (20% manual), in hypoxic chambers (2% manual) or in the automated incubator operating at 2% $O_2$ (2% automated). In the case of 20% manual and 2% manual conditions, medium exchanges were carried out manually in a laminar flow hood operating under atmospheric conditions. Plates being cultured in the 2% automated condition were moved onto the environmentally controlled automation platform for medium exchanges as outlined in the Materials and Methods. We measured the oxygen tension in the liquid phase of culture plates placed in either the cytomat incubator or on the Tecan Evo platform during operation under the 2% automated condition. 2% $O_2$ was

measured in both as shown in Figure 1c. These results confirm that our automation platform was capable of operating under hypoxic conditions during both the cell culture and liquid handing phases of the process.

### 3.1 The effect of automated bioprocessing on neural rosette formation

Neural cells obtained during the directed differentiation of mESC tend to form colonies known as neural rosettes (Ying *et al.,* 2003). In order to investigate the effect of automated culture on the morphology of neural rosettes, cells were fixed after 8 days of differentiation and stained for βIII tubulin expression (Figure 3). These results show that the 2% $O_2$ automated process successfully resulted in the formation of βIII tubulin positive neural rosettes. There was a 3.5 fold increase in rosette density at 2% $O_2$ manual as compared with 20% $O_2$ manual ($p < 0.05$) (Figure 4a). Importantly there was no significant difference between the rosette density or diameter (Figure 4b) at 2% $O_2$ manual compared with 2% $O_2$ automated ($p > 0.05$) confirming that the automation process had not affected the formation of neural rosettes.

### 3.2 The effect of automated bioprocessing on the yield and purity of the process

After 8 days of differentiation under all three conditions cells were detached to analyse the final cell number and viability. The 2% $O_2$ manual process yielded 3 fold more viable cells than the 20% $O_2$ manual process ($p < 0.05$). Similar final cell numbers were obtained for 2% $O_2$ manual and 2% $O_2$ automated processes yielding $2.37 \pm 0.24 \times 10^5$ cells·cm$^{-2}$ and $2.27 \pm 0.18 \times 10^5$ cells·cm$^{-2}$ respectively ($p > 0.05$) (Figure 5a). The

expression of one neural stem cell marker (Nestin) and two neuronal markers (βIII tubulin and MAP2) were assessed by flow cytometry at the end of differentiation protocol. The percentage of cells expressing Nestin and βIII tubulin was very similar when comparing 2% $O_2$ manual and 2% $O_2$ automated processes ($p > 0.05$). The 2% $O_2$ automated process resulted in 21 ± 2 % of the final population expressing MAP2. This represented a 3-fold increase in MAP2 expression as compared with 2% $O_2$ manual ($p < 0.05$) and a 7-fold increase in MAP2 expression when compared to 20% $O_2$ manual ($p < 0.05$) processes (Figure 5b). A similar trend was observed for the cellular density of each neural population, which was calculated by multiplying the final cell number under each condition with the percentage of cells expressing each marker (Figure 5c). There were no significant differences between the density of Nestin and βIII tubulin positive cells when comparing 2% $O_2$ manual with 2% $O_2$ automated processes. A 3 fold increase in density of βIII tubulin positive cells was obtained when 2% $O_2$ manual and 2% $O_2$ automated were compared with the 20% $O_2$ manual condition ($p < 0.05$). The density of MAP2 positive cells in the 2% $O_2$ automated process was 16 fold higher than 20% $O_2$ manual, whilst the density of MAP2 positive cells in the 2%$O_2$ manual condition was 6 fold higher than 20% $O_2$ manual (both $p < 0.05$) (Figure 5C). In summary, these results indicate that the automated platform was capable of achieving similar cell densities to the manual process. The increase in MAP expression may be due to the elimination of transient exposure to 21% $O_2$ during medium exchanges. Finally, it is worth noting that in this current study we only assessed 3 markers. There may be many other oxygen-sensitive lineages which could be present in the final population (i.e. pluripotent cells and

other differentiated lineages). In addition, there may have been significant co-expression between the different markers sets during the ESC differentiation process.

### 3.3 The effect of automated bioprocessing on cell growth

In order to further investigate the impact of environmentally controlled automated medium exchanges we sacrificed a 6 well plate every 2 days to measure cell density and cell viability under all three conditions. At days 6 and 8 of culture the cell density values were significantly lower in the 20% manual processing culture condition, when compared to both hypoxic cultures (Figure 6a). Comparisons of the cell growth curves revealed that the expansion of cells was very similar in both the 2% manual and 2% automated processes although cell growth may have been slightly slower in the manual condition. For further analysis of cellular metabolism we considered the first 4 days of culture as the lag phase followed by an exponential phase from 4 days to 8 days based on the growth curves. The specific growth rates were calculated for the exponential phase as indicated in equation 1 and were found to be, 0.5 ± 2, 0.6 ± 0.2 and 0.4 ± 0.1 $day^{-1}$ for the 2% automated, 2% manual and 20% $O_2$ manual conditions. There were no statistically significant differences between the growth rate of 2% automated and 2% manual $O_2$ culture conditions.

### 3.4 The effect of automated bioprocessing on cell metabolism

In addition to cell growth, cell metabolism was monitored for the three different culture conditions every 2 days during the 8 days of differentiation protocol. Glucose consumption, lactate production, and ammonia concentrations are shown in Figure 6 b-d. A linear decrease in glucose consumption and lactate production was detected over the whole period of culture for all processing conditions with no statistical differences except for day 2, where lactate production was higher in the 2% $O_2$ manual condition, when compared to the other culture modes (Figures 6b and 6c). Specific rates for glucose consumption and lactate production are in agreement with values from the literature [41, 42]. These results indicate that the automation platform did not interfere with glucose metabolism. Analysis of ammonia concentrations (figure 6d) revealed that differentiation at 20% $O_2$ caused the accumulation of this metabolite to potentially toxic levels [49]. Under both 2% $O_2$ manual and automated conditions ammonia levels were remained relatively low in comparison. Lower levels of toxic ammonium may have contributed to the higher levels of viability observed at 2% $O_2$ [33].

## 4 Discussion

Cell culture automation has improved over the years and systems have been integrated into production lines enhancing industrial (i.e. pharmaceutical) production in terms of efficiency and productivity. Barrier isolators and cGMP conditions have been adapted for pharmaceutical and biotechnological processing avoiding cross-contamination and operator exposure [26]. A number of studies have previously demonstrated that commercially available automation platforms can be used to develop robust stem cell production processes based on two dimensional cell culture systems [12,

23-28]. Automated platforms enable accurate and repeatable control of plate handling and liquid handling velocities and forces. This will allow processes to avoid variable exposure to shear stresses, which can influence ESC differentiation [44-46]. In addition, automated technology avoids contact with human operators thus minimizing a potential source of contaminations [47]. In this study we demonstrate that automation platforms can be used for the two dimensional differentiation of ESC. We envisage that the type of closed system we employed could be used for a range of differentiation processes many of which are highly irreproducible and labour intensive. The majority of human ESC neural differentiation processes rely upon two stage processes. Firstly aggregates known as embryoid bodies are grown in suspension before being allowed to attach into two dimensional culture format for final maturation towards the target phenotype. A number of groups have already demonstrated the use of stirred tank bioreactor technologies for the culture of embryoid bodies in the first stage [17, 20]. For this type of process we envisage that automated cell culture platforms could now be used for the second part of the process.

Our bespoke automation platform differs from commercially available systems in its ability to control oxygen tension during both the cell culture and the liquid handling stage. This allowed us to perform automated two-dimensional neural differentiations under oxygen-controlled conditions (2% $O_2$). Physiological oxygen tension surrounding the early embryo during normal development is significantly lower than laboratory oxygen tensions commonly used in most ESC protocols (~20% $O_2$) [33]. Measurements of oxygen tension in the uterus of several mammals ranged from 1.5 to 8.7% $O_2$ [48], while oxygen tensions in the mature brain environment ranging from 0.1% to 5% $O_2$ [49].

Mimicking the in-vivo microenvironment has been a powerful tool for increasing the efficiency of ESC differentiation into a wide range of cell types [15, 20, 32-38, 42]. Therefore, it is important that any new automation platform for ESC processing should have the ability to control this critical parameter. In a previous study we have shown that the yield of neural cells from mESC can be greatly enhanced by manually operating at 2% $O_2$ [33]. However, ESC differentiation processes typically require medium changes every other day (in some cases this can be daily). Using traditional hypoxic chambers the oxygen tension at the growth surface rose to 21% $O_2$ during medium exchanges [33]. It took approximately 5 h for the oxygen tensions at the growth surface to return to 2% $O_2$ once the cell culture dishes had been returned to a hypoxic environment. Our automation platform was designed with this in mind and liquid handling steps were carried out at 2% $O_2$. The data here revealed that our automation platform was capable of delivering a similar process to the manual control. One unexpected result was a 3-fold increase in the number of cells expressing the neuronal marker MAP2. We tentatively hypothesise that the formation of MAP2 positive cells is sensitive to exposure to ambient conditions and that eliminating these shifts resulted in an increase in this population. Our automated process did not affect the formation βIII-tubulin or Nestin positive populations indicating that eliminating transient shifts on oxygen does not have a beneficial impact on all neural lineages. This is in keeping with our previous results showing that the oxygen had a more pronounced impact on the formation of MAP2 positive cells when compared with βIII-tubulin or Nestin [33, 50]. A more detailed time-course analysis of marker expression will be needed in order to further investigate whether the automation process affected the rate of differentiation. Taken together, these results demonstrate that transient shifts in

oxygen tension to ambient conditions may inhibit some of the positive effects associated with differentiation under physiological conditions.

In summary, we have shown for the first time that an automation platform can be used for the neural differentiation of embryonic stem cells in a two dimensional format. This is of particular importance given that many hESC, induced pluripotent stem cell (iPSC) and adult stem cell directed differentiation protocols rely upon this type of attached culture format for the generation of a wide variety of cells for regenerative medicine applications. Our system enables the transfer of labour intensive process, which are highly dependent upon operator to operator variability [39], into controlled, automated bioprocess. In addition, we exploited the system's ability to control oxygen during the liquid handling steps to ensure that $O_2$ levels remained consistent throughout the entire 8 day process.

## 5 Executive Summary

- We have developed a novel automation platform capable of controlling $O_2$ during liquid handling steps.
- The platform was tested on the neural differentiation of mouse embryonic stem under hypoxic conditions (2% $O_2$) resulting in similar cellular yields when compared with the equivalent manual process.
- There was a 3-fold increase in the number of cells expressing the neuronal marker MAP2 in the automated platform.

- Glucose, lactate and ammonia metabolism was not affected by automated processing

**Acknowledgements**

The authors would like to acknowledge the engineering support from Tecan (http://www.tecan.com/) and Walker Safety Cabinets (http://www.walkersafetycabinets.co.uk/) for their support during the design and construction of the automation platform. PMT would like to acknowledge his PhD financial support from the Mexican Science and Technology Agency (CONACYT) and from the Agency of Public Education (SEP). FSV, GJL and CM acknowledge financial support from the Technology Strategy Board (then Department of Trade and Industry).

## REFERENCES

1 Mason C and Hoare M. Regenerative medicine bioprocessing: Building a conceptual framework based on early studies. *Tissue Engineering.* 13(2), 301-311 (2007).

2 McNeish JD. Embryonic stem cells in drug discovery. *Nat Rev Drug Discov.* 3, 70-80 (2004).

3 Thomson H. Bioprocessing of stem cells for drug discovery. *Trends Biotech.* 25(5), 224-230 (2007).

4 McNeish JD. Stem cells as screening tools in drug discovery. *Current Opinion in Pharmacology.* 7(5), 515-520 (2007).

5 Conrad C, Gupta R, Mohan H, *et al*. Genetically engineered stem cells for therapeutic gene delivery. *Curr Gene Ther.* 7(4), 249-260 (2007).

6 Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature.* 292, 154–156 (1981).

7 Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci.* 78, 7634–7638 (1981).

8 Bradley A, Evans M, Kaufman MH and Robertson E. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature.* 309, 255–256. (1984).

9 Thomson JA, Itskovitz-Eldor J, Shapiro SS, *et al*. Embryonic stem cell lines derived from human blastocysts. *Science.* 282, 1145–1147 (1998).

10 Smith AG. 2001. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol.* 17, 435–462 (2001).

11 Kirouac DC, Zandstra PW. The systematic production of cells for cell therapies. Cell *Stem Cell.* 3(4), 369-381 (2008).

12 Thomas RJ, Chandra A, Liu Y, Hourd PC, Conway PP, Williams DJ. Manufacture of a human mesenchymal stem cell population using and automated cell culture platform. *Cytotechnology*. 55, 31-39 (2007).

13 Platas Barradas O, Jandt U, Phan LDM, *et al*. Criteria for bioreactor comparison and operation standardization during process development for mammalian cell culture. *BMC Proceedings.* 5 (8), 47 (2011).

14 Tissot S, Zhang X, Stettler M, *et al*. Engineering principles and cell culture performance of orbitally shaken bioreactors. *ESACT Proceedings.* 5(5), 407-412 (2012).

15 Bauwens C, Yin T, Dang S, Peerani R, Zandstra PW. Development of a perfusion fed bioreactor for embryonic stem cell-derived cardiomyocyte generation: oxygen-mediated enhancement of cardiomyocyte output. *Biotech and Bioeng.* 90, 452-461 (2005).

16 Schroeder M, Niebruegge WA, Willbold E, *et al.* Differentiaton and lineage selection of mouse embryonic stem cells in a stirred bench scale bioreactor with automated process control. *Biotech and Bioeng.* 92(7), 920-933 (2005).

17 Cormier JT, Zur Nieden NI, Rancourt D, Kallos MS. Expansion of undifferentiated murine embryonic stem cells as aggregates in suspension culture bioreactors. *Tissue Eng.* 12(11), 3233-3245 (2006).

18 McLeod M, Hong M, Sen A, *et al*. Transplantation of bioreactor-produced neural stem cells into the rodent brain. *Cell Transplantation* 15, 389- 697 (2006).

19 Fernandes AM, Fernandes TG, Diogo MM, Lobato da Silva C, Henrique D, Cabral JMS. Mouse embryonic stem cell expansion in a microcarrier-based stirred culture system. *Journal of biotechnology.* 132, 227-236 (2007).

20 Niebruegge S, Bauwens CL, Peerani R, Thavandiran N, Masse S. Sevaptisidis E. Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotech and Bioeng* 102, 493–507 (2009).

21 Serra M, Brito C, Costa EM, Sousa MFQ, Alves PM. Integrating human stem cell expansion and neuronal differentiation in bioreactors. *BMC Biotechnology.* 9, 82 (2009).

22 Kehoe DE, Jing D, Lock LT, Tzanakakis SE. Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. *Tissue Eng Part A.* 16(2), 405-421 (2010).

23 Dang SM, Gerecht-Nir S, Chen J, Iskovitz-Eldor J, Zandstra PW. Controlled scalable embryonic stem cell differentiation culture. *Stem Cells.* 2(3), 275-282 (2004).

24 Narkilahti S, Rajala K, Pihlajamaki H, Suuroen R, Hovatta O, Skottman H. Monitoring and analysis of dynamic growth of human embryonic stem cells: comparison of automated instrumentation and conventional culturing methods. *Biomedical Engineering online.* 6,11 (2007).

25 Terstegge S, Laufenberg I, Pochert J, *et al*. Automated Maintenance of Embryonic Stem Cell Cultures. *Biotech and Bioeng.* 96(1), 195-201 (2007).
** Describes the first automated platform for the culture of mouse and human embryonic stem cells using enzymatic methods for cell passaging.

26 Thomas RJ, Anderson D, Chandra A, *et al*. Automated, scalable culture of human embryonic stem cells in feeder – free conditions. *Biotech and Bioeng.* 102 (6), 1636 – 1644 (2009).
*Reports an automated platform for the culture of human embryonic stem cells using enzymatic method for cell passaging and matrigel to avoid the use mouse feeder cells.

27 Thomas RJ, Hope AD, Hourd P, *et al*. Automated, serum-free production of CTX0E03: a therapeutic clinical grade human neural stem cell line. *Biotech Lett.* 31(8), 1167-1172 (2009).

28 Thomas RJ and Williams HDJ. Application of process quality engineering techniques to improve the understanding of the in vitro processing of stem cells for therapeutic use. *Journal of Biotechnology*. 136(3), 148-155 (2008).

29 Forristal C, Wright K, Hanley N, Oreffo R, Houghton F. Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. *Reproduction.* 139, 85–97 (2010).

30 Forsyth NR, Musio A, Vezzoni P, Simpson AH, Noble BS, McWhir J. Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. *Cloning Stem Cells.* 8, 16–23 (2006).

31 Ezashi T, Das P, Roberts RM. Low O2 tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci.* 102, 4783–4788 (2005).

32 Han Y, Kuang SZ, Gomer A, Ramirez-Bergeron DL. Hypoxia influences the vascular expansion and differentiation of embryonic stem cell cultures through the temporal expression of VEGF-receptors in an ARNT-dependent manner. *Stem cells.* 28, 799-809 (2010).

33 Mondragon-Teran P, Lye GJ, Veraitch FS. Lowering oxygen tension enhances the differentiation of mouse embryonic stem cells into neuronal cells. *Biotechnology progress.* 25(5), 1480-1488 (2009).
** Describes the impact of controlling oxygen at physiological levels during the neural differentiation of embryonic stem cells.

34 Morrison SJ, Csete M, Groves AK, Melega W, Wold B, Anderson DJ. Culture in reduced levels of oxygen promotes clono- genic sympathoadrenal differentiation by isolated neural crest stem cells. *J Neurosci*. 20, 7370–7376 (2000).

35 Studer L, Csete M, Lee SH, *et al*. Enhanced proliferation, survival, and dopaminergic differentiation of CNS precursors in lowered oxygen. *J Neuro- sci.* 20, 7377–7383 (2000).

36 Milosevic J, Schwartz S, Krohn K, Poppe M, Storch A, Schwartz J. Low atmospheric oxygen avoids maturation, senescence and cell death of murine mesencephalic neural precursors. *J Neurochem*. 92, 718–729 (2005).

37 Pistollato F, Chen H, Schwartz P, Basso G, Panchision D. Oxygen tension controls the expansion of human CNS precursors and the generation of astrocytes and oligondendrocytes. *Mol Cell Neurosci.* 35, 424–435 (2007).

38 Bae D, Mondragon-Teran P, Hernandez D, *et al*. Hypoxia enhances the generation of retinal progenitor cells from human induced pluripotent and embryonic stem cells. *Stem Cells and Development,* 21(8): 1344-1355 (2012).

39 Veraitch FS, Scott R, Wong J, Lye GJ, Mason C. The impact of manual processing on the expansion and directed differentiation of embryonic stem cells. *Biotech and Bioeng.* 99, 1216– 1229 (2008).

*Describes how poorly controlled **manual** cell culture techniques can affect the expansion and differentiation of embryonic stem cells.

40 Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. *Nat Biotech.* 21, 183–186 (2003).

*Developed and reported a method for the neural differentiation of mouse embryonic stem cells in monolayer format.

41 Fernandes TG, Diogo MM, Fernandez-Platzgummer A, da Silva CL, Cabral JM. Different stages of pluripotency determine distinct patterns of proliferation, metabolism, and lineage commitment of embryonic stem cells under hypoxia. *Stem Cell Res.* 5, 76–89 (2010).

42 Rodrigues CA, Diogo MM, da Silva CL, Cabral JM. Hypoxia enhances proliferation of mouse embryonic stem cell-derived neural stem cells. *Biotech and Bioeng.* 106, 260–270 (2010).

43 Butler M. Animal cell culture and technology, 2nd Ed. New York: BIOS Scientific (2004).

44 Illi B, Scopece A, Nanni S, *et al*. Epigenetic histone modification and cardiovascular lineage programming in mouse embryonic stem cells exposed to laminar shear stress. *Circulation Research.* 96(5), 501- 508 (2005).

45 Zeng L, Xiao Q, Margariti A, Zhang Z, Zametaki A, Patel S, Capogrossi MC, Hu Y, Xu Q. HDAC3 is crucial in shear – and VEGF induced stem cell differentiation toward endothelial cells. *The journal of cell biology.* 174 (7), 1059 – 1069 (2006).

46 Stolberg S, McCloskey KE. Can shear stress direct stem cell fate? *Biotechnology Progress.* 25 (1), 10-19 (2009).

47 Dutton RL and Fox JS. Robotic processing in barrier isolator environments: A life cycle cost approach. *Pharmaceutical Engineering.* 26 (5), 1-8 (2006).

48 Fischer B and Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil.* 99, 673–679 (1993).
** Reports the physiological oxygen tension values in the oviduct of different mammals where neurulation occurs during organogenesis.

49 Andreasen A, Danscher G, Juhl SS, *et al*. Distinct differences in partial oxygen pressure at micrometer ranges in the rat hippocampal region. *J Neurosci Methods*. 72, 15-21 (1997).

50 Mondragon-Teran P, Baboo JZ, Mason C, Lye GJ, Veraitch FS. The full spectrum of physiological oxygen tensions and step-changes in oxygen tension affects the neural differentiation of mouse embryonic stem cells. *Biotechnology progress.* 27(6), 1700-1708 (2011).

**FIGURE LEGENDS**

**Figure 1.** The automation platform used for the neural differentiation of mESC. (a) Photograph of liquid handling robot contained within a Class-II biosafety cabinet and integrated with and automated incubator and microplate centrifuge. (b) Photograph of the liquid handling plaform: RoMA (i), Te-Link (ii), tilting plate holder (iii), sterile media reservoir (iv), multi pipetting liquid handling arm (v) and sterile tips (vi). (c) Oxygen tension measurements at the growth surface of 6 well plates containing 2 ml of NDIFF-RHBA on the liquid handling platform and within the Cytomat incubator during operation at 2% $O_2$.

**Figure 2.** Standard Operation Procedure (SOP) for automated neural differentiation of mESC. The cell culture and media renew steps were performed by Cytomat incubator and Tecan EVO automated platform respectively.

**Figure 3.** βIII tubulin staining for neural rosettes obtained from 20% manual, 2% manual and 2% automated processes after 8 days of differentiation. From left to right: bright field/phase contrast, DAPI staining for cell nuclei and βIII tubulin immunocytochemistry. Scale bars represent 100 μm.

**Figure 4.** Quantification of neural rosette formation after 8 days of differentiation. Rosette density per $cm^2$ (a) and average diameter of neural rosettes (b) formed at 20% manual, 2% manual and 2% automated processes were evaluated from a total of 30 fields of view taken from three independent wells. Rosettes that expressed βIII tubulin were assessed with the aid of image processing analysis in Java (Image J, NIH, USA). Error bars represent one standard deviation about the mean of three independent data points (n = 3). Statistical analysis has been indicated as $p < 0.05$ (*).

**Figure 5.** The yield and purity of neural cells produced after 8 days of differentiation under 20% manual, 2% manual and 2% automated conditions. (a) Viability and viable cell number after 8 days of differentiation and (b) The percentage of cells expressing Nestin, βIII-tubulin and MAP2 measured by flow cytometry. (c) represents the fold increase in the number of cells.$cm^2$ expressing Nestin, βIII-tubulin and MAP2 at 2%

manual and 2% automated as compared with 20% manual controls. Error bars represent one standard deviation about the mean of three independent data points (n = 3). Statistical analysis has been indicated as $p < 0.05$ (*).

**Figure 6.** Cell growth and metabolism during the automated neuronal differentiation of mESC. Growth curve for manual and automated conditions showing total viable cell number (a), specific glucose consumption (b) and lactate production (c) rates, in $pmol.cell^{-1}day^{-1}$, and the cumulative production of ammonia (d), in mM, measured every 2 days for 20% manual culture (■), 2% manual culture (●) and 2% automated culture (▲). Error bars represent one standard deviation about the mean of three independent data points (n = 3).

**Supplementary Figure 1.** Immunocytochemistry analysis of pluripotency marker expression in E14Tg2a cells. The left panel shows brightfield images and the right panel shows DAPI (Blue) merged with Oct3/4 (green) (a), Nanog (green) (b). Scale bar represents 100 μm.

**Supplementary Figure 2.** Immunocytochemistry analysis of βIII tubulin and MAP2 expression after 8 days of differentiation at 2% $O_2$ (manual condition). The images are at 40X magnification showing βIII tubulin (green) and DAPI (blue) in (a) and MAP2 (green) and DAPI (blue) in (b). Scale bar represents 10 μm.