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Reproducible culture and differentiation of mouse embryonic stem cells using an automated microwell platform☆

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ABSTRACT

The use of embryonic stem cells (ESCs) and their progeny in high throughput drug discovery and regenerative medicine will require production at scale of well characterized cells at an appropriate level of purity. The adoption of automated bioprocessing techniques offers the possibility to overcome the lack of consistency and high failure rates seen with current manual protocols. To build the case for increased use of automation this work addresses the key question: "can an automated system match the quality of a highly skilled and experienced person working manually?" To answer this we first describe an integrated automation platform designed for the 'hands-free' culture and differentiation of ESCs in microwell formats. Next we outline a framework for the systematic investigation and optimization of key bioprocess variables for the rapid establishment of validatable Standard Operating Procedures (SOPs). Finally the experimental comparison between manual and automated bioprocessing is exemplified by expansion of the murine Oct-4-GiP ESC line over eight sequential passages with their subsequent directed differentiation into neural precursors. Our results show that ESCs can be effectively maintained and differentiated in a highly reproducible manner by the automated system described. Statistical analysis of the results for cell growth over single and multiple passages shows up to a 3-fold improvement in the consistency of cell growth kinetics with automated passaging. The quality of the cells produced was evaluated using a panel of biological markers including cell growth rate and viability, nutrient and metabolite profiles, changes in gene expression and immunocytochemistry. Automated processing of the ESCs had no measurable negative effect on either their pluripotency or their ability to differentiate into the three embryonic germ layers. Equally important is that over a 6-month period of culture without antibiotics in the medium, we have not had any cases of culture contamination. This study thus confirms the benefits of adopting automated bioprocess routes to produce cells for therapy and for use in basic discovery research.

1. Introduction

Embryonic stem cells (ESCs) are currently being evaluated for potential application in a number of diverse areas including regenerative medicine [1], drug discovery and development [2–4] or 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 almost every somatic cell type [6–10].

Effective exploitation of ESCs is, however, predicated upon the ability to reproducibly derive, manipulate and efficiently differentiate these cells on a suitable scale at an appropriate level of purity [11–15]. The often reported lack of reproducibility in ESC processing is a consequence of the large number of processing steps involved, generally carried out using largely uncontrolled and manual operations. Previous work in our laboratory and elsewhere has shown that ESCs are particularly sensitive to the microenvironment prevalent during processing [11,16–18]. For example, the effect of physical forces on cells due to fluid flow during pipetting was shown to be highly significant resulting in a large degree of culture variability among similarly trained operators. Automation of ESC handling offers the potential to reduce such variation since applied physical forces may be tightly controlled and applied consistently throughout a culture, and from culture to culture [19].

A number of initial publications on the automation of aspects of ESC processing have appeared in the last few years. The first was that of Joannides and co-workers [20] who modified an existing

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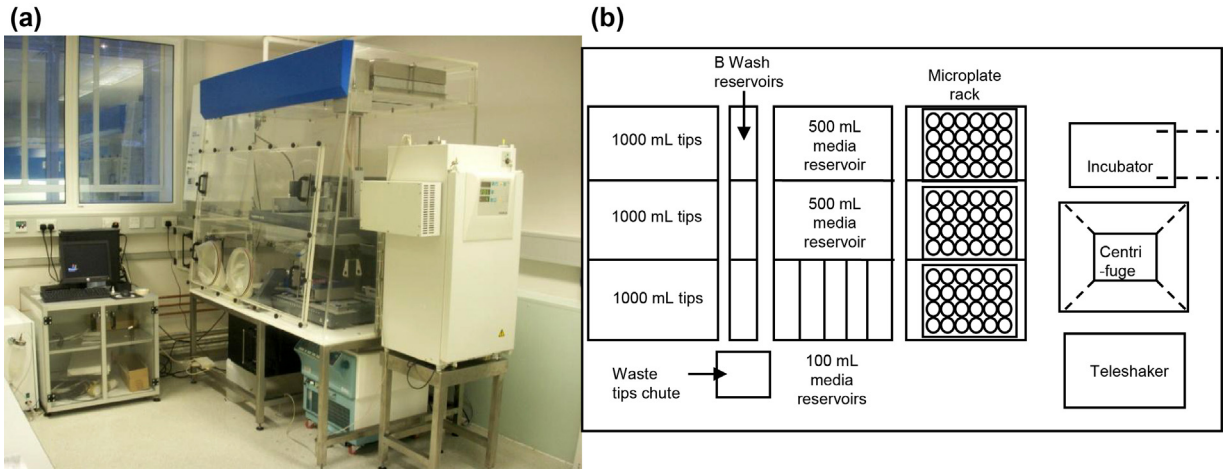

Fig. 1. Automation platform for the reproducible culture and differentiation of mouse embryonic stem cells. (a) Photograph of liquid handling robot contained within the Class-2 design biosafety cabinet. (b) Schematic layout of the robotic deck showing access (dashed lines) to the integrated CO_2 incubator (plate conveyor belt) and microplate centrifuge (via the Tecan RoMa arm).

'tissue chopping device' to create an instrument for the automated mechanical passaging of human ESCs. This work was the first to address the pressing need for standardization of routine, and often variable laboratory work with a view to processing stem cells at scale for therapy. The use of automated liquid handling to address the issue of standardization of physical forces resultant of fluid flow has also been reported [19]. In this case the plating out of both human and murine ESCs using a liquid handling robot was reported along with automated media exchanges. Complete automated passaging of ESCs was precluded by the lack of an integrated centrifuge to facilitate removal of cell dissociating agents. Automated expansion of ESCs in traditional T-flasks has also been reported [21]. It was shown that ESCs can be routinely maintained and expanded in an automated T-flask system yielding cell numbers at the scale required for therapeutic applications. Most recently, murine ESCs have been expanded in microplates on an automated liquid handling platform and further differentiated into cardiomyocytes by embryoid body (EB) differentiation on a separate microfluidic liquid handling workstation [22], which is optimal for high-throughput screening. Manual transfer of cells was required however between the expansion and differentiation stage. Furthermore, none of the platforms described incorporation of the key centrifugation step to facilitate enzymatic passaging of cells. To date then, there remains no report in the literature on the complete, hands-free automation of all key steps in the processing of ESCs including trypsinization, centrifugation and cell differentiation.

In this work, we address a key question related to the use of stem cells in drug discovery and future development of the regenerative medicine industry [12]: 'Can an automated system match the quality of a highly skilled and experienced person working manually?' Consequently, we describe an integrated automation platform (Fig. 1(a)) for the 'hands-free' expansion and differentiation of ESCs. Related to this is a framework for the systematic investigation and optimization of key bioprocess variables in order to define validatable Standard Operating Procedures (SOPs) for automation platform operation. The comparison between automated and manual processing is exemplified by the maintenance of the murine ESC line Oct-4-GiP [23], in 24-well microtitre plates, over eight sequential passages. The subsequent automated and directed neural differentiation of these ESCs is also demonstrated. Our results show that ESCs can be effectively maintained and differentiated in a highly reproducible manner by the automated system described.

2. Materials and methods

2.1. Routine cell culture and maintenance

The mouse embryonic stem cell line Oct-4-GiP [23] was used throughout this work and was kindly provided by Stem Cell Sciences (Cambridge, UK). The cells were cultured under feeder-free conditions on Iwaki tissue culture treated plastic (SLS, Nottingham, UK) coated with 0.1% (w/v) gelatine (Sigma, Poole, UK). Routine maintenance of the cells was carried out in T-flasks and all other experimental work was carried out in 24-well microplates. For undifferentiated cell growth the culture medium consisted of Glasgow Minimum Essential Medium (GMEM) supplemented with 0.1 mM β-mercaptoethanol, MEM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine (all Invitrogen, Paisley, UK), 10% (v/v) foetal bovine serum (SLS, Nottingham, UK) and 1×10^3 U mL^{-1} leukemia inhibitory factor, LIF (Chemicon, UK). All stated concentrations are final. Cell dissociation was routinely achieved by removal of the culture medium followed by washing with Dulbecco's PBS (DPBS, Sigma, Poole, UK) and subsequent incubation for 4 min at 37 °C in the presence of 0.025% (w/v) trypsin supplemented with 0.372 g L^{-1} EDTA and 1% (v/v) chicken serum (all Sigma, Poole, UK). Following incubation the trypsinized cells were quenched using fresh culture medium and centrifuged for 3 min at $280 \times g$ in an Eppendorf 5810R centrifuge in the case of manual cultures or in a Hettich Rotanta 46 RSC centrifuge in the case of automated culture. Following centrifugation and supernatant removal, the cells were resuspended in growth medium and replated onto a fresh gelatinized microtitre plate at the desired Inoculation Cell Density (ICD). Cultures were maintained in a humidified incubator at 37 °C with 5% (v/v) CO_2.

2.2. Directed monolayer neuronal differentiation

ESCs were cultured manually for 2 days in serum containing medium in the presence of LIF as described in Section 2.1. Seeding at 3×10^4 cells cm^{-2} in a T-25 flask typically yields around 6×10^6 cells after 48 h. Differentiation was performed in Iwaki 6-well plates, the wells previously being gelatinized for around 1 h with 0.1% (w/v) gelatine at room temperature and then the excess gelatine removed. The cells were harvested by incubation with 0.025% (w/v) trypsin (containing 1% (v/v) chick serum) for ~4 min as described in Section 2.1 and quenched with serum containing