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Embryoid body arrays: Parallel cryosectioning of spheroid/embryoid body samples for medium through-put analysis

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ABSTRACT

Three dimensional (3D) culture of mammalian cells is emerging as a powerful new tool to understand organogenesis as well as serve as models for diseases with implications for therapeutic evaluation. 3D cultures are referred to variously as spheroid, organoids or embryoid bodies. While many methods exist for large scale production of embryoid bodies or other spheroid cell aggregates, either at controlled sizes using microwell/micropatterned plates or uncontrolled sizes in suspension dishes, very few protocols exist for medium through-put analysis of differentiation at the histological level. We have developed a method which allows for parallel processing, sectioning and analysis of multiple 3D samples (*e.g.* fixed at different time points, treated with different drugs/growth factors, generated from different cell lines etc.) by double-embedding blocks in a larger array format. Our protocol has few barriers for use and requires only materials commonly found in any lab currently using embedding materials for cryosectioning. Sectioning in parallel allows histological techniques (such as histochemistry, immunostaining or *in situ* hybridisation) to be performed simultaneously on many samples on a single slide. This reduces slide to slide variation as well as requiring less reagents, fewer consumables with lower time and labour requirements when compared to individually embedded samples.

1. Introduction

One of the emerging new approaches in stem cell, developmental and cancer cell biology is the ability to generate three dimensional (3-D) *in vitro* cultures which better mimics adult organs and provides for a more physiologically relevant model for understanding developmental processes, disease mechanisms and also high throughput drug screening (Huch and Koo, 2015). Mouse and human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) when cultured in a 3-D environment form structures called embryoid bodies (EBs) which can also be directed to form organ specific structures called organoids (Huch and Koo, 2015). Tissue specific adult stem cells can also form 3-D organoids when cultured in appropriate growth conditions (Huch and Koo, 2015). Cancer cell lines such as from breast cancer also can be grown in 3-D to form spheroid cultures.

The advent of reliable and low cost isolation methods for pluripotent stem cells leads to an increased need for thoroughly characterising their pluripotent status through differentiation and the status of derived differentiated cells through their ability to form EBs which contain cell types derived from all three germ layers (Takahashi and Yamanaka, 2006). The need for medium to high throughput analysis increases rapidly with increased numbers of clonal cell lines required to provide biological replicates of control and mutant genotypes, cell lines isolated from multiple siblings or kindred, or multiple alleles within the same gene of interest. Furthermore it is generally hard to predict the point during differentiation at which a defect of interest to the researcher will occur, necessitating observation at multiple time points. All of these points conspire to produce large numbers of experimental samples which are both time- and reagent-consuming in nature. Such large number of samples can also arise when drug testing is carried out in 3-D organoid or cancer cell derived spheroid cultures.

The ability of ES and iPS cells to form EBs is a very common approach used to characterise embryonic and induced pluripotent cells (Ader and Tanaka, 2014; Buta et al., 2013). EBs arise when ES or iPS cells are allowed to aggregate in suspension in the absence of pluripotency maintaining factors. The resulting EBs differentiate in a fashion similar to that seen in the early embryo, forming the three germ layers and associated tissues (Desbaillets et al., 2000; Kerr et al., 2006).

Many methods have been described to generate EBs, the simplest of which involves plating cells into suspension dishes and allowing aggregation to occur spontaneously. This results in EBs which are highly variable in size, this in turn leads to variations in the direction of differentiation within each EB formed (Kurosawa, 2007). EB size has been shown to have a substantial influence on differentiation and many modern methods utilise EBs formed from defined numbers of cells (Carpenedo et al., 2010; Karp et al., 2007). These were typically generated through labour intensive 'hanging drop' methods however

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many modern methods use microwell plates or micropatterned surfaces for high-throughput formation of controlled size EBs (Miyamoto et al., 2016; Sakai et al., 2011).

Histological techniques such as histochemistry, immunostaining or *in situ* hybridisation are commonly used techniques for identifying the lineages of cells present within an EB. Samples are typically embedded separately in embedding medium and sectioned serially onto slides. This leads to the need to work with multiple slides carrying sections from each experimental condition/sample for each round of histology. To circumvent this, we have adapted the technique of tissue arrays developed for probing several precious human tissue samples in a single slide and applied it to EBs from multiple experimental samples. This enables us to generate a single slide carrying sections from a single arrayed block. While tissue arrays are typically wax blocks, our EB array consists of double-embedded OCT supported frozen material and is sectioned using a cryo-microtome.

EBs can be stained in whole-mount but clear results are harder to obtain without resorting to confocal microscopy. Moreover, in whole mount staining more material is used overall from experimental samples (Carpenedo et al., 2009) which then is a disadvantage if experimental material is limiting. In addition, whole mount is more used more frequently when performing *in situ* hybridisation of embryos (Piette et al., 2008). Microtome sections of EB arrays on slides provide a very simple approach to subject EBs to these techniques. EB arrays permit the visualisation of histological details of multiple EBs from a single time point or treatment. They also allow simultaneous comparison of multiple EBs from different time points or treatments under comparable conditions. Another advantage is that the same EB can be stained using different antibody markers/insitu probes/dyes in adjacent sections thus increasing the amount of material available from each experiment. Sections from an EB array can be stained by using histochemical techniques, immunostaining or *in situ* hybridisation.

Here we describe the development of a method for generating EB arrays from mouse R1 ES cell derived (a) EBs formed in serum containing media and (b) neuronally directed EBs formed in serum free medium. We show its application in histochemical staining as well as immunostaining. The EB array method can be also applied to organoids or spheroids.

2. Materials and methods

2.1. Cell culture and generation of embryoid bodies (EBs)

The mouse ES cell line R1 was routinely maintained on a feeder layer of MITC fibroblasts in mES medium (DMEM with glutamax, 10% FCS, 5% KOSR, 1% NEAA and 0.1 μM 2-mercaptoethanol). Prior to EB formation, the R1 ES cell line was adapted to feeder free conditions and cultured in mES medium supplemented with LIF (1000 μ/ml) on gelatinised plates and passaged at 48 h intervals by trypsinsation with a 1:10 split ratio.

EBs were generated from ES lines by plating 2.5×10^5/ml of feeder independent cells in 10 ml of EB medium (DMEM + Glutamax containing 5%KOSR, 5%FCS, NEAA and 0.1 μm β mercaptoethanol) in 10 cm bacteriological. EBs were cultured over a period of 20 days with media changes every 48 h. EBs were harvested for embedding at indicated time points.

R1 EBs were generated as above by plating on bacteriological petri dishes and Retinoic acid (0.5 μM) was added to the EBs 24 h after plating to induce neuronal differentiation. On day two EBs were transferred to alpha-MEM containing glutamax, NEAA, 0.1% KOSR, 0.5 μM RA (RA differentiation medium). Differentiation media was changed at 48 h intervals. EBs were fixed at required time points in 4% PFA in PBS for 1 h and processed for embedding as described below.

2.2. Embedding EBs for generating tissue arrays

EBs were collected and fixed under conditions suitable for the desired analysis, in our case this is typically 4% PFA or Methanol-Acetic acid. The first steps are performed in 15 ml centrifuge tubes to allow for a great excess during washes. After thoroughly washing away fixative with 3–5 washes of 10 min each PBS if necessary (Fig. 1C,D), EBs are infiltrated overnight with cryoprotectant 30% sucrose (Sigma) in PBS (Oxoid) (Fig. 1E), tubes are incubated on their side a 4 °C with gentle shaking. Due to the viscosity of the OCT we have found that both OCT and EBs suspended in it stick considerably to the inside of plastic micropipette tips but not to glass. Hence we use glass Pasteur pipettes with flame polished tips which are made prior to starting to process the EBs further. Wide-bore, flame polished pipettes are made as follows- glass Pasteur pipettes are cut with a diamond tipped pen where the neck narrows for a bore of 3–4 mm (Fig. 1A), and very briefly exposed to a blue Bunsen flame to smoothen the sharp edges (Fig. 1B).

Twenty four hours after incubation in 30% sucrose, the tubes with the EBs are set upright and EBs allowed to settle (Fig. 1F). As much of the sucrose as possible is then aspirated and a volume of OCT (Lamb) is added- roughly five times that of the EB pellet. Wide-bore flame-polished pipettes are used with a small plastic bulb to gently mix the EBs the OCT (Fig. 1G) without creating air bubbles. If there are very few small EBs they can be left to settle overnight (Fig. 1H). Small cylindrical moulds are prepared for each sample by cutting a 1 ml syringe barrel into 1 cm lengths using a razor blade. One end of the cylindrical moulds is sealed with a small square of parafilm (Fig. 1I). Using the wide-bore flame-polished glass pipettes, around ~200 μl of OCT is taken up from above the EB pellet to act as a buffer and then the tip is then taken further into the OCT with the EBs and EBs gently taken up into the pipette. The EBs are then dispensed into the cylindrical mould starting from the base of the mould and moving up so at to put the majority of EBs at the cutting face of the block (Fig. 1K). A metal plate or block is placed on dry ice and allowed to cool. The cylindrical moulds with the EBs are placed on the cold metal plate and allowed to freeze. Once frozen the parafilm is removed and the cylinder moulds stored in 1 ml microfuge tubes at −80 °C until ready to process further to create the blocks with multiple cylindrical embedded EBs.

An outer mould large enough to take up to 9 cylinders can be prepared from a Shandon Peel-A-Way® mould. A truncated, 22 mm square top, tapered to 12 mm bottom is cut at the start of the taper, with the taper taking up to four cylinders and the square upper portion taking up to nine (Fig. 1J). OCT is added to form an even 1–2 mm layer at the bottom of the Peel away mould and kept at room temperature. Frozen cylinder blocks are popped out of plastic cylinders using the plunger from a 1 ml syringe (Fig. 1L) and placed in labelled chilled microfuge tubes. The required number of cylindrical moulds with EBs are placed in the Peel-A Way mould with OCT using pre-chilled forceps and the whole mould onto the rapid freezing area of the cryostat (Fig. 1M). Once the inner blocks are frozen securely in place, the larger mould is filled to the top with OCT. A 1 ml micropipette tip placed over the OCT bottle nozzle can be used to fill in the small gaps. The blocks are allowed

Fig. 1. Stepwise creation of an EB section array. Glass pipettes are cut to give a wide bore (A) and flame polished (B). EBs are initially processed in 15 ml centrifuge tubes (C) and allowed to settle (D) between reagent changes.Upon addition of the 30% sucrose as a cryo-protectant, aggregates float initially (E). Further processing is done when the EBs have sunk in the sucrose solution (F). EBs are gently mixing in OCT due to its viscosity (G) and allowed to settle (H). Primary embedding moulds made from the barrel of a 1 ml syringe pressed onto parafilm (I). Larger secondary moulds for embedding multiple EB blocks in an array (J), A Shandon tapered peel-away mould (left) is split at the taper creating two moulds; four blocks fit in the tapered mould (middle) while up to nine blocks can be arranged in the square mould on a base of masking tape (right). EBs in OCT are pipetted from the base up into the primary moulds to ensure concentration near the cutting face at the base (K). After freezing, primary blocks are released and arranged in the cryostat (L). Primary blocks are pressed into the secondary mould with a thin layer of OCT in the base, and allowed to freeze in place (M). After further addition of OCT to the secondary mould and freezing to create a solid block, the completed array can be sectioned (N), mounted on subbed slides and briefly air-dried (O).