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Scalable cell alignment on optical media substrates

Chukwuemeka G. Anene-Nzelu [a], Deepak Choudhury [b,c], Huipeng Li [d], Azmall Fraiszudeen [a], Kah-Yim Peh [a], Yi- Chin Toh [e], Sum Huan Ng [b], Hwa Liang Leo [a,**], Hanry Yu [c,d,e,f,g,h,*]

[a] Department of Bioengineering, National University of Singapore, Block EA, #03-12, 9 Engineering Drive 1, Singapore 117576, Singapore
[b] Singapore Institute of Manufacturing Technology, 71 Nanyang Drive, Singapore 638075, Singapore
[c] NUS Graduate School for Integrative Sciences and Engineering, Centre for Life Sciences (CeLS), #05-01, 28 Medical Drive, Singapore 117456, Singapore
[d] Singapore-MIT Alliance for Research and Technology, 1 CREATE Way, #04-13/14 Enterprise Wing, #B-10, Singapore 138602, Singapore
[e] Institute of Bioengineering and Nanotechnology, A*STAR, #04-01, 31 Biopolis Way, Singapore 138669, Singapore
[f] Mechanobiology Institute, Temasek Laboratories, National University of Singapore, #05-01, 5A Engineering Drive 1, Singapore 117411, Singapore
[g] Department of Physiology, Yong Loo Lin School of Medicine, National University Health System, MD9, 2 Medical Drive, Singapore 117597, Singapore
[h] Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

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ABSTRACT

Cell alignment by underlying topographical cues has been shown to affect important biological processes such as differentiation and functional maturation *in vitro*. However, the routine use of cell culture substrates with micro- or nano-topographies, such as grooves, is currently hampered by the high cost and specialized facilities required to produce these substrates. Here we present cost-effective commercially available optical media as substrates for aligning cells in culture. These optical media, including CD-R, DVD-R and optical grating, allow different cell types to attach and grow well on them. The physical dimension of the grooves in these optical media allowed cells to be aligned in confluent cell culture with maximal cell–cell interaction and these cell alignment affect the morphology and differentiation of cardiac (H9C2), skeletal muscle (C2C12) and neuronal (PC12) cell lines. The optical media is amenable to various chemical modifications with fibronectin, laminin and gelatin for culturing different cell types. These low-cost commercially available optical media can serve as scalable substrates for research or drug safety screening applications in industry scales.

1. Introduction

Cells in many organs are presented with different topographical features by the respective extracellular matrix (ECM) in their basement membrane [1]. The basement membrane consists of ECM components such as glycosaminoglycans, fibrous proteins like fibronectin and collagen, growth factors and cytokines anchored on ECM fibers, hyaluronic acid, laminin etc. displaying unique features of pores, fibers and ridges in the scale of nanometers [2]. The arrangement of these ECM molecules presents morphological and differentiation cues to the cells lying on them. A good example is the heart *in vivo* which is a highly anisotropic organ, and the cardiomyocytes in the heart are aligned because of the parallel arrangement of the collagen fibers [3].

Just as these topographical cues regulate biological processes *in vivo*, presenting cells with topographical features *in vitro* can also affect cellular morphology and differentiation capabilities [4]. Cell alignment *in vitro* can be achieved either through micropatterning ECM molecules into line geometries [4] or by creating grooves and ridges on the substrates [3]. Published reports have shown that grooves and ridges with dimensions ranging from 35 nm to 25 μm in width and 14 nm to 5 μm in depth can induce cell alignment [5,6]. This cell alignment has been used for various applications such as engineering muscle tissues [7], stem cell differentiation [8], mechanobiology studies [9], cell proliferation [10,11], and ECM production [12]. In an interesting study, researchers have shown that topographical cues from micro-grooved substrates alone were sufficient to direct the switch of stem cells towards a particular cell fate such as neuronal or myogenic pathways without the aid of specific induction growth factors [13,14]. Other cell types such as cardiomyocytes and skeletal myocytes exhibit a more mature

* Corresponding author. Department of Physiology, Yong Loo Lin School of Medicine, National University of Health System, MD9 #04-11, 2 Medical Drive, Singapore 117597, Singapore.
** Corresponding author.
E-mail addresses: bielhl@nus.edu.sg (H.L. Leo), hanry_yu@nuhs.edu.sg, medyuh@nus.edu.sg, hyu@ibn.a-star.edu.sg, hanry_yu@nus.edu.sg (H. Yu).

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functional phenotype when they are aligned in culture [15–17]. Replicating this aligned cellular structure *in vitro* can therefore be used for applications such as models to study differentiation, for functional electrophysiological studies of the cardiac muscle [18], and as *in vitro* ensembles for pharmacological studies and drug screening platforms [19].

Current technologies used to fabricate micro/nanogrooved substrates for cell culture include: photolithography, electron beam lithography, nanoimprint lithography, electrospinning and UV embossing [5,20]. Besides the high cost of fabrication, these techniques involve lengthy procedures, specialized clean room facilities, highly skilled labor and are technically challenging to scale up to large surface areas [21]. Hence, despite the various advantages of aligning cells in culture, the challenges greatly limit the translation of interesting findings relating cell alignment to cell function into practical and routine cell culture applications.

Here, we propose that commercially available optical media such as CD-R, DVD-R, and optical gratings, present a readily available source of nano/micro-grooved substrates that fulfills the needs of aligning cells in a cost-effective manner. A standard size CD-R or optical grating costing between 1 and 5 USD can produce enough micro-grooved cell culture inserts for seven 24-well plates. The materials of the optical media, polycarbonate (CD-Rs and DVD-Rs) and polyester (optical gratings), have been shown to support cell culture [22,23], and the dimensions fall within the published range for producing cell alignment *in vitro* [5]. Although some forms of the optical media have previously been used to pattern other polymers for cell culture [24,25], there has been no report on direct culturing and alignment of cells on these optical media. Therefore we demonstrate here that the optical media can directly support cell attachment, growth, alignment and differentiation. Commercially available optical media can therefore be exploited as a scalable and cost-effective source of micro-grooved cell culture substrates. This will allow researchers to incorporate cell alignment for routine culture of cardiac, skeletal or neuronal cells to support cell and tissue research and drug testing applications.

2. Materials and methods

2.1. Cell lines

All the cell lines used were below passage 20. They include 3T3 fibroblasts, H9C2, C2C12 and PC12 cells from ATCC. HL-1 was received as a kind gift from Prof Williams Claycomb, Louisiana State University, New Orleans, USA.

2.2. Processing of CD-R/DVD-R/and optical gratings for cell culture

Three examples of commercially available optical media include CD-R (Imation, Singapore), DVD-R (Verbatim, Singapore) and optical grating (Edmund optics, Singapore). For the CD-Rs, the label, acrylic and the aluminum layers on top were peeled off with an adhesive tape to expose the polycarbonate layer. For the DVD-R, we peel off the top cover layer to expose the grooved polycarbonate layer in the middle for further processing and cell culture. The exposed polycarbonate surfaces of CD-Rs/DVD-Rs were cut into pieces (1 cm × 1 cm), so that they can fit in 24-well plates. For the optical gratings, 13 mm diameter pieces were punched out using a metal punch (Helmold, Ilinois USA). CD-R/DVD-R and optical grating pieces were then treated with absolute methanol for 1 h, sonicated for 30 min and then rinsed with DI water to remove any dust particles and chemicals, especially the organic dyes in case of optical discs. The optical media pieces were sterilized by treating with 70% ethanol for 1 h, and rinsed with sterile DI water or autoclaved at 105 °C for 21 min. The optical media pieces were then placed in a well plate to complete "Gratings in a dish" device. The substrates will be made available through Bio-Byblos (Taiwan, ROC) as Vivoalign™.

2.3. Surface characterization by Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM)

SEM/AFM samples were prepared by cutting appropriate sizes of the optical media. For SEM, the samples were viewed with a JSM 5600 scanning electron microscope (Jeol, Japan) at 5 kV. Prior to imaging, the gratings were sputter-coated with platinum for 60 s. For CD-R samples with 0.1% gelatin, the protein was fixed by glutaraldehyde and serially dehydrated with ethanol before SEM was done. Atomic force microscope, DI Nanoscope Dimension 3100 (Digital Instruments, USA) was used in tapping mode to identify the groove features on the optical media.

2.4. Nano indentation to determine Young's modulus

The mechanical test for the gratings was performed with a nano-indentation system [27] at ambient temperature. Superglue was used to attach specimen to the stage. A Berkovich diamond indenter with depth-control method was used during the nano-indentation test. The maximum depth was fixed at 1 μm for indentation tests across all the samples. Both loading and unloading rate were 0.1 mN/s and the dwelling time at the maximum load was 60 s. Each indent was of size 100 μm.

2.5. Plasma treatment to render the surface hydrophilic

The CD-R/DVD-R and optical grating cell culture inserts were plasma treated for 3 min using the plasma machine (FEMTO, CUTE-B, South Korea) and then soaked in ethanol for 30 min. After the ethanol treatment, they were rinsed three times with PBS before coating with ECM for experiments with PC12 cell lines.

2.6. Water contact angle measurement of the surface

Five microliter of DI water was pipetted on the gratings surface. Water contact angles were measured with a goniometer (Contact Angle System OCA 30, Data Physics Instruments GmbH, Germany) using the SCA20 software.

2.7. ECM coating of CD-R/DVD-R and optical gratings

After plasma treatment, 10 μg/ml of laminin (354232, BD biosciences, Singapore) was used to coat the gratings for 1 h in the case of PC12 cells. A solution of Fibronectin (F1141, Sigma–Aldrich, Singapore) and Gelatin (G1890, Sigma, Singapore) was used for HL-1 cells while the other cell types (H9C2, 3T3 and C2C12) did not need ECM coating.

2.8. Picogreen assay

H9C2 cells were seeded at a density of 13,000 cells/cm^2 and allowed to attach for 1 h on the tissue culture plastic (TCP) and both the grooved and flat surfaces of the optical media used. After 1 h, unattached cells were washed off with PBS; the optical media pieces were taken to new wells and cells were lysed with 0.1% SDS, 500 μL per well. The assay was performed with the Quanti-iT™ PicoGreen® dsDNA kit (P11495, Invitrogen, USA). A standard curve was established with known cell number of H9C2 cells.

2.9. Alamar blue assay

H9C2 cell growth over 5 days was monitored using the alamarBlue® Cell Viability Assay Protocol (DAL1100, Invitrogen, Singapore). Cells were incubated with 10% alamarBlue® in culture media (vol/vol) for 2 h on days 1, 3 and 5 and the fluorescence was measured with the Infinite M1000 plate reader (Tecan, Switzerland) with absorption wavelength at 560 nm and emission wavelength at 590 nm. Fluorescence intensity values for days 3 and 5 were normalized to the fluorescence intensity for day 1 as an indication of the relative cell number compared to the number of attached cell on day 1.

2.10. RNA isolation, cDNA synthesis and qPCR analysis

Cells were lysed using RLT buffer from Qiagen. Cell lysate was collected and RNA isolation was done and total RNA was reverse transcribed to cDNA according to manufacturer's protocol. Custom designed primers for cardiomyocytes (Supplementary Table 1) and already published primers for C2C12 cells [17] were used for the quantitative PCR and the reactions were performed using both Roche lightcycler and Bio-Rad real time system. Analysis of each gene was performed using the relatively quantitative ΔΔCT method. Transcript levels were first normalized to the housekeeping gene GAPDH and expressed as relative level to that on the flat surface.

2.11. Differentiation of H9C2 cells

The H9C2 is a cell line derived from embryonic rat heart tissue and is widely used to study the rat physiology and cardiotoxicity. The cell line can differentiate into both skeletal and cardiac muscle. Upon addition of all-trans retinoic acid and reduction of serum content, the H9C2 cell differentiates into cardiac lineage [28]. The H9C2 cells were seeded at a density of 13,000 cells per cm^2 containing 1 cm × 1 cm square pieces of CD-R and DVD-R. After the cells attained confluence about 2 days after seeding, the cells were treated with 1 μM of all-trans retinoic acid (R2625, Sigma Singapore) daily for 5 days. At the end of the 5 days, the RNA isolation and gene expression was carried out and cells fixed for immunostaining.