Introduction

Human induced pluripotent stem cells (hiPSCs)\textsuperscript{1,2} can now be efficiently generated from multiple sources of somatic cells and be subsequently differentiated to produce various cell types of the human body. This technology is now beginning to realize significant promise for research and the development of potential cell therapies. This technology allows disease modeling and drug screening to be carried out using diverse population cohorts and with more relevant cell phenotypes than can be accommodated using traditional immortalized cell lines. However, technical complexities in the culture and differentiation of hiPSCs, including lack of scale and standardization and prolonged experimental timelines, limit the adoption of this technology for many large-scale studies, including personalized drug screening. The entry of reproducible end-to-end automated workflows for hiPSC culture and differentiation, demonstrated on commercially available platforms, provides enhanced accessibility of this technology for both research laboratories and commercial pharmaceutical testing. Here we have utilized TECAN Fluent automated cell culture workstations to perform hiPSC culture and differentiation in a reproducible and scalable process to generate patient-derived retinal pigment epithelial cells for downstream use, including drug testing. hiPSCs derived from multiple donors with age-related macular degeneration (AMD) were introduced into our automated workflow, and cell lines were cultured and differentiated into retinal pigment epithelium (RPE). Donor hiPSC-RPE lines were subsequently entered in an automated drug testing workflow to measure mitochondrial function after exposure to "mitoactive" compounds. This work demonstrates scalable, reproducible culture and differentiation of hiPSC lines from individuals on the TECAN Fluent platform and illustrates the potential for end-to-end automation of hiPSC-based personalized drug testing.

Keywords

human induced pluripotent stem cells, automation, automated cell culture platform, retinal pigment epithelium, age-related macular degeneration, personalized drug screening, TECAN Fluent, liquid handling
Standardized reagents and protocols for hiPSC derivation and maintenance culture under conditions compliant for both good laboratory practice (GLP) and current good manufacturing practice (cGMP) are being rapidly adopted worldwide in academia and industry. Conventional adherent human pluripotent stem cell cultures requiring feeder cells and media containing undefined serum or xenobiotic products have been superseded by feeder and xenobioc-free, fully defined culture media and culture substrates. These conditions have enabled the development of robust, reproducible, and economic methods for manual hiPSC culture and provide the basis for improved standardization of hiPSC culture, as well as making the culture of hiPSCs amenable to automation.

Although there have been advances in batch culturing of human pluripotent stem cells for scaled production of cells, most current differentiation protocols for hiPSCs still require an adherent culture format for all or part of the process. The basis of adherent hiPSC culture is repetitive liquid handling procedures involving aspirating and dispensing various volumes of media, media supplements, or cell suspensions. The benefits of automated cell culture workflows include improving precision and reproducibility, the reduction of labor costs, and increasing experimental scale. In addition, proof-of-principle demonstrations of processes performed on commercially available equipment allow immediate dissemination of both methods and laboratory practice beyond the initiating institution. Automated cell cultures paired with automated imaging allows culture assessment and data acquisition over extended time periods, including both real-time measurements and endpoint assays that can be integrated into workflows.

In this paper, we have utilized commercial TECAN Fluent workstations to demonstrate the automated culture of multiple hiPSC lines and automated differentiation of multiple iPSC lines into retinal pigment epithelium (RPE) and their application in a personalized drug testing paradigm. RPE is a single layer of pigmented cells at the base of the retina that supports the health and function of the light sensing retinal neurons. Diseases and genetic conditions that compromise the health or function of the RPE can result in loss of retinal neurons and vision impairment. RPE derived from human embryonic stem cells (ESCs) and iPSCs has also entered clinical trials around the world to test safety and efficacy as a cell replacement therapy for macular diseases. We have been building on previous reports of automating retinal differentiation from hiPSCs to convert manual protocols for iPSC and RPE differentiation and develop automated procedures for hiPSC culture and iPSC-RPE differentiation that use fully defined media, substrates, and differentiation molecules and can be conducted on the new generation of commercially available robotic cell culture workstations. This work is designed to increase the scale of generating donor- or patient-specific lines of iPSC-RPE for clinically relevant populations. Increasing the cost-effective scaling of iPSC-RPE derivation and drug testing will support the implementation of personalized drug screening and population-based disease modeling for patients with AMD.

Materials and Methods

TECAN Fluent 780 Workstation

This work utilizes TECAN Fluent 780 robotic workstations, purchased by the Department of Ophthalmology and Visual Neurosciences and installed at the Laboratory for Stem Cell Automation located in the University of Minnesota (UMN) Stem Cell Institute (Minneapolis, MN). The units comprise TECAN Fluent 780 base units and cabinets (TECAN, US, Morrisville, NC) with HEPA and UV hoods (Bigneat Ltd., Waterlooville, Hampshire, UK). The units are each customized with an eight-tip Air Flexible Channel Arm (FCA); a Multiple Channel Arm (MCA) with a 96-well head adaptor; an extended z-rail Robotic Gripper Arm (RGA) with finger exchange system (FES) eccentric fingers, FES centric fingers, FES tube fingers, and the FES docking station; a LiCONiC StoreX STX44 ICSA (LiCONiC US INC., Woburn, MA), an Incubator linked by a transporter; an Integrated Hettich High Speed Robotic Centrifuge (Hettich, Tuttinglen, Germany) positioned below the deck level; two EchoTherm RIC20 Dry Baths (Torrey Pines Scientific, Carlsbad, CA); and a Cytation 1 high-content imager (BioTek, Agilent Technologies, Santa Clara, CA). The TECAN software used is Fluent Control version 2.4.25.51907 (TECAN Trading Ltd., Switzerland).

hiPSC Lines

The hiPSC lines used in this study are listed in Table 1. The derivation and characterization of hiPSC lines from individuals with and those without age-related macular degeneration (AMD) utilized in this study have been previously described.20 Line UMN PCBC-16ips (lab designation 9-1) was previously derived from neonatal human dermal fibroblasts (ATCC PCS 201-010).21

Manual hiPSC Culture

Manual hiPSC culture was performed essentially as previously described. Briefly, undifferentiated hiPSC cultures were maintained with Essential 8 media (Thermo Fisher Scientific A1517001) on tissue culture dishes coated with recombinant human vitronectin (Peprotech AF-140-09). Cells were passaged using hypertonic citrate solution. Cells
were released using gentle trituration with media and collected with centrifugation at 300 g for 5 min. Supernatant was discarded and cells resuspended in fresh media. Media was changed daily.

**Automated hiPSC Culture on UMN Fluent 780 Workstations**

Custom TECAN Fluent Control scripts were optimized to automate the culture of hiPSC lines in a six-well adherent format, adapting our manual culture system described in Geng et al. Daily media exchange consisted of the following steps. The culture plate was retrieved from the Liconic incubator and transferred to the Cytation 1 imager where a preset Gen5 protocol was executed to image selected wells and report the confluence to an Excel file. The plate was then positioned on the tilt carrier. The plate lid and the media trough cover were relocated to the hotel and the tilt carrier moved to an angle of −5°. The FCA collected 1000 µL of filtered SBS pipette tips (TECAN 30057817) and media aspiration occurred from the lowest position of each well. Used media was disposed in the liquid discard trough and used tips in the trash; new tips were collected. Fresh media was collected from the media trough and dispensed into the wells; the plate lid and media trough cover were replaced and the tilt carrier rocked to distribute media. The tilt carrier then returned to 0° and the plate was moved to the transport position and returned to the incubator.

For passaging, a vitronectin-coated plate was placed in the first position of the tilt carrier. The passaging script can passage a single well or two wells simultaneously. The plate designated for passage was retrieved from the incubator and placed onto the tilt carrier, where the lid was removed and the tilt carrier assumed an angle of −5°. The FCA used 1000 µL of filtered SBS tips to aspirate media and 1 mL of hypertonic citrate solution was added to each well. The tilt carrier was tilted back and forth to coat the well, the solution was aspirated from the wells, and another 1 mL of hypertonic citrate solution was dispensed to each well. The plate was rocked and the lid replaced before the plate was transported to the 37 °C incubator for 6 min. During the incubation time, 1 mL of media was dispensed into each well that would receive cells. After incubation, the plate was recalled from the incubator and 1000 µL of filtered wide bore SBS tips (TECAN 30115239) were used to detach and collect the cells. Cell aspiration used a designed liquid handling microscript that uses aspiration and dispensing 12 times in a pattern across each well to detach cells before aspirating from the lowest point of the well. The cell suspension was dispensed into a 15 mL conical tube and 1 mL of media was dispensed into each well (to disperse any remaining adhered cells) using a modified microscript with six aspirations and dispensing steps across each well before the media was added to the cell suspension. An additional 3 mL of media was then also added into the 15 mL tube. Tubes containing the cell suspension, and balance tubes if needed, were moved with the RGA into the below-deck centrifuge, set in opposing positions, and centrifuged at 800 g for 3 min before retrieval and repositioning on the tube rack. Using 1000 µL of filtered SBS tips, the FCA removed the supernatant from the 15 mL tube aspirating 5.5 mL and discarded the media in the liquid discard trough. After equipping new tips (six standard and one wide bore), a total of 6 mL of media was added incrementally onto the cell pellet. First, 1 mL was added, and then the wide bore tip was mixed three times to disrupt the pellet. This was followed by an additional 2 mL, where the mixing

<table>
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<tr>
<td>UMN PCBC-16iPS (9-1)</td>
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<td>UMN MGS1-0237-3A3</td>
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<td>UMN MGS2-1747-1C1</td>
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<td>UMN MGS3-1424-1A4</td>
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NA, not applicable. *Minnesota Grading System. **Derived from primary neonatal dermal fibroblasts (ATCC PCS-201-010). ***Cell lines derived from the same donor. ****Cell lines derived from the same donor.
process was repeated and repeated again when the final 3 mL was added. At that point, the cell pellet was fully distributed throughout the media. The wide bore tip then aspirated a set volume of the cell mixture and distributed it into wells on the new plate. The split ratio was user defined for cell line maintenance, expansion, or distribution for differentiation. The lids were placed back onto the plates and the media trough, and the new plate was moved into the transport position. Before being returned to the incubator, the RGA lifted and shook the plate three times in the x direction, twice in the y direction, and then twice more in the x direction.

**Automated RPE Differentiation of hiPSC Lines**

The 14-day RPE differentiation of hiPSC lines was conducted essentially as described previously utilizing the TECAN workstation to exchange the media components automatically over the 2-week differentiation period for 5 hiPSC lines simultaneously. iPSC cells were plated on vitronectin-coated plates, and starting on day 1 of differentiation, the media components were exchanged daily. Stock solutions of the reagents for RPE differentiation were prepared and loaded onto the TECAN workstation distributed in V-bottom 96-well plates (Sarstedt 82.1583.001). The plates were kept frozen at −20 °C on the EchoTherm RIC20 Dry Baths. Basal neural induction media (DMEM/F12 with 1× N2 supplement [Life Technologies 17502-048], 1× B27 supplement [Life Technologies 17504-044], and 1× nonessential amino acids) was prepared by diluting the supplements in DMEM/F12 media. This media was used to thaw and resuspend the appropriate reagents on each day of the differentiation. The reagents were compounded daily in a 15 mL conical tube and DMEM/F12 was added to achieve the correct media concentration. The media was then changed on the appropriate wells. On days 1–4, the media was supplemented with 10 mM Nicotinamide (Sigma-Aldrich N0636), 50 ng/mL Noggin (R&D Systems 6057-NG), 10 ng/mL Dkk-1 (R&D Systems 5439-DK), and 10 ng/mL insulin-like growth factor 1 (IGF-1; R&D Systems 291-G1). On days 3 and 4, the Noggin concentration was reduced to 10 ng/mL and basic fibroblast growth factor (bFGF) was added at 5 ng/mL (R&D Systems). On days 5 and 6, the basal media was supplemented with 10 ng/mL Dkk-1 (R&D Systems 5439-DK), 10 ng/mL IGF-1 (R&D Systems 291-G1), and 100 ng/mL Activin A (PeproTech AF-120-14E). Then, from day 7 to day 14, the basal media was supplemented with 100 ng/mL Activin A and 10 µM SU5402 (Sigma-Aldrich SML0443), with 3 µM CHIR 99021 (Tocris, Bristol, UK, 4423) added in addition from day 8 to day 14.

**Expansion and Maturation of iPSC-RPE**

Fourteen-day RPE derived from hiPSCs was cultured with XVIVO-10 media (Lonza 04-743Q) on six-well plates (CytoOne, USA Scientific, CC7682-7506) treated with vitronectin XF (Stem Cell Technologies 07180). Passaging was carried out by exposure to Accumax (Sigma-Aldrich A7089) dissociation reagent for 25 min at 37 °C, and the cells were detached with a cell scraper and centrifuged for 5 min at 200g. Cells were resuspended in XVIVO-10 containing 10 µM ROCK inhibitor Y27632 (Cayman Chemical 10005583) with subsequent media changes of XVIVO-10 without inhibitor. Cells were passed through a 40 µm cell strainer (Corning 07-201-430) before plating at 1:3–1:4 split ratios (1 × 10^6 cells/cm^2). Cells were incubated at 37 °C with 5% CO₂ with twice weekly media changes for at least 30 days.

**Workstation Consumables**

Workstation consumable use is dependent on the workflow program. Pipetting steps using the eight-tip FCA utilized ANSI format, filtered, conductive tips in 1000 µL (TECAN 30057817) and 10 µL (TECAN 30104974) sizes. Autoclaved 1000 µL wide bore (TECAN 30115239) tips were primarily used in steps involving cell suspension pipetting. Pipetting with the MCA96 utilized nested, sterile, nonfiltered, 200 µL tips (TECAN 30038619). Disposable tubes and troughs were utilized on the worktable, including 15 mL conical tubes (Sarstedt 62.554.002) and 300 and 100 mL media troughs (TECAN 10613048 and 30077312); Autoclavable custom lids were manufactured in house for these. Cells for this work were grown in three plate formats, 6-well (CytoOne CC7682-7506), 96-well V-bottom (Sarstedt 82.1583.001), and Seahorse XF96 Cell Culture Microplates (Agilent 101085-004).

**Immunocytochemistry**

Cells were fixed in buffered formalin (10% w/v; Fisher Scientific 23-305-510) for 10 min at room temperature, permeabilized for 10 min at room temperature in Dulbecco’s phosphate-buffered saline (DPBS) with 0.2% v/v Triton X-100 (Millipore Sigma T8787); and blocked in blocking buffer (DPBS with 1% w/v bovine serum albumin [BSA; Millipore Sigma A3059] and 0.1% v/v Tween-20 [Millipore Sigma P1379]) for 1 h. Cells were then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. Cultures were washed once with blocking solution and incubated 1 h with secondary antibodies diluted in blocking buffer. Nuclei were labeled with DAPI (4′,6-diamidino-2-phenylindole dilactate) (Thermo Fisher D3571) for 10 min before washing two times in DPBS. The primary antibodies used were NANOG (1:100, BioTechne AF1997), OCT4 (1:500, Millipore Sigma MAB4401MI), PMEL17 (1:200, DAKO M063429-2), OTX2 (1:250, Abcam ab21990), and ZO1 (1:500, Invitrogen 61-7300). The secondary antibodies used were Alexa Fluor 488 Donkey Anti-Mouse (1:500, Thermo Fisher Scientific A21202), Alexa

Automated Imaging and Image Analysis

Cells were imaged using the Cytation 1 and analyzed using the Gen5 data analysis software (BioTek, Agilent Technologies). The Cytation 1 was equipped with 10× and 20× objective lenses and green fluorescent protein (GFP) and DAPI imaging filter cubes. For each well, 64 images were taken in an 8 × 8 square with each picture spaced 1500 µm apart. For each channel capture, exposure was set to “Auto” and kept consistent between samples. Unstained cultures or cultures with secondary antibodies only were used to establish negative expression. Each image was autofocused using the DAPI channel within a 10-micron offset for the fluorescent image from the appropriate secondary antibody images. For ZO1 imaging, using the Gen5 software, a primary mask was applied to the image to determine the center of each cell marked by DAPI, and a secondary mask using the fluorescent image of the secondary antibody. For nuclear epitope imaging (OCT4, NANOG, and OTX2), the DAPI primary mask was applied and a subpopulation was created for all cells where expression in the appropriate secondary antibody channel was also detected.

“Mitoactive” Drug Testing

Eighteen compounds were selected from literature reports of mechanisms of action that improved mitochondrial function (see Table 2). Aliquots of concentrated stock solutions were prepared and stored at −20 °C. iPSC-derived RPE cells were seeded into a 96-well Seahorse plate using the Fluent workstation at a density of 4 × 10^4 cells/well and cultured in MEM alpha media (Gibco) containing 5% FBS (Hyclone), sodium pyruvate, 1× Glutamax, 1× nonessential amino acids, 1× N1 supplement, taurine (0.25 mg/mL), hydrocortisone (0.02 µg/mL), and 1× pen-strep at 5% CO₂ and 37 °C. The media was switched to 1% FBS 2 days prior to treatment. On the day of screening, the Fluent workstation was utilized to prepare 500 µM solutions of each drug by adding media to the frozen stock plates (N-acetyl-l-cysteine was prepared fresh) (see Suppl. Fig. S1C), and cells were treated with each drug at a final concentration of 10 µM for 2 days in MEM alpha media with 1% FBS prior to Seahorse assay.

Measuring RPE Mitochondrial Function Using the Seahorse Cell Mito Stress Test

The mitochondrial function of iPSC-RPE was measured using an XFe96 Extracellular Flux Analyzer (Agilent Technologies) and the Cell Mito Stress Test (CMST) assay conditions described previously.24 After incubating iPSC-RPE cells with drugs or solvent only (control) for 48 h, cells were washed (2×) with CMST assay media (XF base media...
DMEM supplemented with 2 mM glutamine, 5.5 mM glucose, and 1 mM sodium pyruvate, pH 7.4) and then incubated in CMST media for 1 h at 37 °C in a non-CO₂ incubator. The CMST assay protocol was performed according to the manufacturer’s instructions (Agilent Technologies). The oxygen consumption rate (OCR) was detected in cells pretreated with either drugs or solvent (control), followed by the sequential addition of oligomycin (2 μM), trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP) (1 μM), and finally rotenone (1 μM) and antimycin A (1 μM). The resultant measurement of OCR allowed for calculation of basal respiration, ATP production, and maximal respiration. Hoechst dye was added in the third and final injection to enable a postassay cell count at 10× magnification using a Cytation 1 imager. Data processing used Wave software version 2.6.1.56 (Agilent Technologies), normalizing OCR to the cell count.

Results

Description of the TECAN Fluent Hardware Platform

The goal of this paper was to demonstrate that commercially available automated workstations can be used to perform complex cell culture workflows and functional assays with hiPSCs. The UMN Laboratory for Stem Cell Automation at the University of Minnesota Stem Cell Institute is equipped with two TECAN Fluent 780 units. The Fluent units can be customized to meet customer specifications with multiple optional components and a wide range of peripheral instruments that can be integrated with the integral workstation components and managed with the TECAN Fluent control software. Figure 1 shows the layout of Fluent 780 workstations installed at the UMN Laboratory for Stem Cell Automation. The workstation layout and range of optional peripheral instruments were planned and integrated in consultation with TECAN engineers to provide a flexible workstation designed to be capable of a wide range of mammalian cell culture procedures with the inclusion of imaging, centrifugation, and long-term cell incubation (Fig. 1A). The UMN Fluent 780 worktable (78 x 165 cm²) (Fig. 1A) has a tilt carrier with locations for up to four plates for controlled angled tilting of plates and shaking. Two EchoTherm dry bath devices provide temperature control between −20 and 110 °C. A storage area is available for short-term plate and lid storage. Optional trough runners are available for 100 mL troughs, 50 and 15 mL conical tubes, and microcentrifuge tubes. About 25% of the deck space is available to pre-position racked pipette tips. Each UMN workstation is equipped with a Liconic STX44 automated incubator programmed to maintain 37 °C, 85% humidity, and 5% CO₂, where two interchangeable incubator racks each hold either 11 tissue culture plates of 6-, 12-, 24-, or 48-well formats or twenty-two 96-well plates for a total cell culture surface area of approximately 1250 cm². A Cytation 1 imager utilizing Gen5 software is integrated for bright-field and fluorescent image acquisition and analysis. The UMN Fluent 780 machines are equipped with three robotic arms (Fig. 1B). The eight-channel air displacement liquid handling arm (air LiHa) allows pipetting of liquids at a volume range from 0.5 to 1000 μL/channel. The MCA is equipped with a 96-channel pipetting head adapter that allows simultaneous pipetting of liquids into microplate formats. The extended robotic manipulator arm (RoMa) is able to transfer plates and other labware on the worktable and to peripheral devices, including transferring tubes to and from the high-speed Hettich robotic centrifuge situated below the worktable. An integrated laminar flow HEPA hood and workspace enclosure maintain air quality to reduce contamination of deck components.

Building an Automated Process to Derive iPSC-RPE for Personalized Drug Screening

A workflow is being developed using automation where possible and advantageous, to generate a scalable process to derive iPSC lines and differentiate and utilize iPSC-RPE from multiple individual donors. Our research has utilized somatic conjunctival cells obtained by manual biopsy. The conjunctival cells grown from the donor biopsies can be manually reprogrammed into iPSCs and differentiated into iPSC-RPE using fully defined manual methods as described previously.20 We are now generating a suite of Fluent control scripts (Fig. 2) that could be linked to form an end-to-end process automating iPSC derivation, culture, and RPE differentiation. When combined with downstream drug screening, this work is intended to increase the scale at which we are able to generate personalized drug screening data for multiple individuals. Processes we have now successfully demonstrated using automation are shown in green in Figure 2, and we are now working to link the automated processes where advantageous and appropriate to improve the workflow. In this current study, we automated iPSC line expansion for banking (not shown) and consecutive seeding of undifferentiated iPSCs for automated RPE differentiation. Separately, iPSC-RPE lines were plated, cultured, and exposed to drug compounds using the automated workstations prior to determining the OCR using the Agilent Seahorse XFe96 Flux Analyzer (see Fig. 5). Further details of the automated workflows are shown in Supplemental Figure 1.

hiPSC Maintenance Culture

A component of all processes using hiPSCs is the maintenance of undifferentiated iPSC lines. This step is critical for successful hiPSC line derivation, undifferentiated iPSC line maintenance during expansion, and characterization prebanking,
during recovery postcryopreservation, and in preparation for differentiation protocols. Using the TECAN Fluent workstations described above, we simultaneously cultured eight different hiPSC lines over expansion and passage cycles using conditions designed to maintain iPSCs with undifferentiated phenotypes (Fig. 3). We adapted previously published cGMP-compliant defined conditions for manual adherent hiPSC culture\textsuperscript{22,25} for the TECAN system. For the demonstration described in Figure 3, cells from eight different previously derived iPSC lines were thawed and cultured in defined Essential 8 media on plates coated with recombinant vitronectin with complete media changes every day. Using automation, we simultaneously passaged each line every 3 days with hypertonic citrate reagent using workflows controlled by custom TECAN Fluent control software scripts (see Suppl. Fig. 1A). Cells were initially seeded to achieve a 24 h postplating colony confluency between 5% and 20% of the available culture area to maintain discrete colony isolation and expanded in culture with daily media changes for two further days before passage. Five consecutive passage cycles were performed with
brightfield imaging of each iPSC line culture conducted each day at the time of media exchange to monitor culture appearance, colony growth, and confluence. Representative brightfield images of expanding iPSC colonies for one line during one passage cycle are shown in Figure 3A. Brightfield images and confluence data are stored digitally and available to the workstation user. Culture confluence for the different iPSC lines cultured simultaneously for 15 days over five passage cycles is shown in Figure 3B. This monitoring of growth kinetics can be used to modify workflow script parameters for individual cell lines as necessary during culture runs. To determine if each cell line had maintained an undifferentiated phenotype following automated culture, immunohistochemistry was used to detect the expression of pluripotency-associated transcription factors NANOG and OCT4 in cells at passages 1 and 5 (Fig. 3C). Quantification of NANOG and OCT4 expression indicated that each cell line retained nuclear expression of these pluripotency-associated proteins, consistent with maintaining an undifferentiated phenotype.

Automated Differentiation of hiPSCs into RPE

After successfully automating the undifferentiated culture of multiple hiPSC lines, we extended the workflow to demonstrate automated differentiation of six of the hiPSC lines into RPE (Fig. 4). We utilized a published 14-day defined differentiation protocol23 that we had previously successfully implemented in manual culture. The protocol utilizes the timed addition of growth factors and small molecules to adherent cells and is suitable for transition to automated simultaneous differentiation of multiple hiPSC lines (Fig. 4A). Aliquots of growth factor small molecules appropriate for each timed addition to the culture wells were prepared, aliquoted, and frozen in a light-protected 96-well plate that was positioned on one of the temperature-controlled deck positions. For this demonstration, hiPSCs from six selected lines were passaged onto vitronectin-coated wells and cultured in Essential 8 media in order to maintain an undifferentiated phenotype until differentiation was initiated. On each day of the differentiation, the appropriate aliquots of differentiation factors were resuspended by addition of media, and the required volumes were introduced to cells during the daily media change as illustrated in Figure 4A. After day 14, differentiated cells from each line were fixed for analysis by immunohistochemistry (Fig. 4B) or continued in manual culture in X-VIVO10 media as described previously.20 Consistent with published reports of manual differentiation using this protocol, hiPSCs differentiated using the automated differentiation workflow were seen by day 14 to have formed a cobblestone monolayer morphology, with cells expressing the tight junction protein ZO1, homeobox transcription factor OTX2, and pre-melanosome protein PMEL17 (Fig. 4C). These characteristics are indicative of the transition to an RPE phenotype. As published previously,20,23 functional RPE can be maintained following extended culture in defined X-VIVO10 media. We conducted the differentiation in a 6-well plate format, 9.6 cm²/well.

Drug Testing with Donor-Specific iPSC-RPE

The TECAN Fluent workstation is especially capable of executing in vitro drug testing and screening protocols involving the exposure of adherent cells to different compounds. These protocols require the plating of cells in appropriate formats, controlled exposure to soluble compounds, and an appropriate functional test. We have previously utilized metabolic analysis to determine the OCR, using the Agilent Seahorse XFe96 Flux Analyzer to examine changes in mitochondrial function in primary RPE from donors with and donors without AMD. To extend this work to iPSC-RPE derived from multiple individual donors graded for the stage of AMD, we employed the TECAN Fluent workstation to conduct iPSC-RPE cell culture, plating, and compound addition for subsequent functional analysis after exposure to different drugs. iPSC-RPE differentiated from
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three different individual donors was plated in the 96-well format and cultured for 48 h before exposure to a targeted library of 18 compounds shown previously to affect mitochondrial metabolism (Table 2). The cells were exposed to the compounds at a standard 10 µM final concentration for 2 days before analysis (Fig. 5). Three metabolic measurements (basal respiration, maximal respiration, and ATP production) were calculated from direct measurement of OCR in the Seahorse Flux Analyzer. For each compound, the mean OCR from duplicate experiments performed on different days is shown as the ratio between cells exposed to the compound and cells exposed to the solute vehicle for three different cell lines. Drugs with a response over 1 were deemed beneficial.

Results from each of the three donor lines are shown arranged from lowest to highest response to drug exposure for basal respiration, maximal respiration, and ATP production (Fig. 5). The raw Seahorse OCR data for these experiments are available from the authors upon request. Overall, donor lines 1B1 and 1A3 were more responsive to drug exposure than donor line 1A2, where the response to all drugs was minimal. Each donor line exhibited an individualized response to the different drugs. For example, clonidine (green boxes in Fig. 5), an α2-adrenoreceptor agonist previously used to treat glaucoma, had a positive effect on basal respiration and ATP production in donor lines 1B1 and 1A3, but had an unfavorable response in donor line 1A2. Exposure to amiodarone (blue boxes in Fig. 5)
demonstrated improvement of basal respiration and ATP production in line 1B1 and basal respiration in line 1A3, but had the most detrimental reduction on all three measured parameters for line 1A3. Of note, some of the drugs also seemed to have a differential effect on the mitochondrial parameters in individual lines. For example, in considering the mitochondrial response to metformin (red boxes in Fig. 5), ATP production was slightly impacted in line 1B1, but improvements in both basal and maximal respiration were observed for this line.

Although results from only three donors are shown here, similar variabilities of response to drugs by individual iPSC-RPE lines have been noted in other studies. We have also seen a variable drug response in primary RPE cultures from donors with AMD. This highlights the importance of testing the drug response of RPE derived from individual donors, and accomplishing this task at a clinically relevant scale will require the adoption of automated processes such as that demonstrated here.

**Discussion**

The successful deployment of automated robotic cell culture systems will be critical to realizing the clinical and commercial potential of human pluripotent stem cell technology. Cell reprogramming to generate donor-specific iPSC lines, which can then be differentiated into specific cell types, provides the ability to pursue disease modeling, drug testing, or therapeutic applications at the level of both individual or large populations in a manner that has not previously been possible. Technological improvements in the culture and differentiation of human pluripotent stem cells, with the introduction of defined culture media, substrates, and small-molecule differentiation reagents, have allowed for the design of
Figure 5. Donor-specific drug testing with hiPSC-RPE. hiPSC-RPE lines from three different donors with (MGS3) or without (MGS1) AMD were cultured in the presence of active compounds or solute vehicle for 48 h before OCRs were determined using a Seahorse XF Flux Analyzer. Results calculated for basal respiration, maximal respiration, and ATP production for each cell line after exposure to each drug are presented as the ratio of the calculated values determined for treated cells or vehicle. The results for two separate experiments are presented for each cell line (squares). The abbreviation for each drug is shown at the bottom. Responses to clonidine (green), metformin (Red), and amiodarone (blue) are highlighted for each line.
reproducible protocols that can be converted to workflows for the new generations of cell culture workstations that have begun to enter the commercial sector. The successful use of these machines can reduce the cost and number of skilled bench technicians, improve reproducibility, and greatly increase sample capacity. This current work demonstrates the use of the TECAN Fluent automated cell culture platform in conducting maintenance culture, differentiation, and a drug screening protocol using hiPSCs. The combination of a fully defined iPSC maintenance culture system with a defined RPE differentiation protocol allowed us to demonstrate the generation of RPE from multiple individual donor iPSC lines for use in a downstream drug screening process. This successful validation of a standardized, hiPSC culture and RPE differentiation process demonstrates a practical approach for using commercial automation equipment for applications requiring the cost-effective generation of RPE from multiple individuals.

The use of automated systems for human pluripotent stem cell culture and differentiation is beginning to gain traction as the potential to increase scale and reproducibility while reducing personnel costs becomes apparent. A number of both custom-built and commercial machines have now been demonstrated (e.g., NYSF Global Stem Cell Array, TAP Biosystems, and Beckmann Coulter I series) for various applications, including pioneering work deriving hiPSC-derived retinal cells on the TECAN Freedom EVO platform or differentiating and expanding RPE from hESCs in the flask-based CompacT SelecT automation platform from Sartorius.

For our current application, we are developing automated protocols to assist research that includes the derivation of hiPSC lines from multiple individuals, both with and without AMD, and the subsequent differentiation of these cells into RPE for disease modeling and drug screening. We have adapted manual culture and differentiation processes that use fully defined reagents and substrates to build automated protocols that can be executed in plate-based adherent workflows on the TECAN Fluent platform. The hiPSC maintenance and expansion protocol employing Essential 8 media and recombinant vitronectin-coated plates, combined with passaging with hypertonic citrate, results in a straightforward protocol that is highly reproducible between cell lines. We utilized a 3-day passage cycle for simplicity, where the daily confluence measurement allows the user to adjust split ratios on passage to maintain optimal confluence. Multiple cell lines can be cultured simultaneously, and this allows coordinated initiation of RPE differentiation for batches of donor lines. The rapid RPE differentiation process was initially described by Buchholz et al. in 2013 and efficiently differentiates iPSC-RPE from most hiPSC lines tested in 14 days. These cells are expanded and matured over 30-day passage cycles in defined X-VIVO10 media. Using the six-well plate format adopted here and seeding undifferentiated iPSCs at 15%–30% confluence prior to RPE differentiation, we generated approximately $15 \times 10^6$ iPSC-RPE cells at P0 for expansion. Each 96-well Seahorse plate requires $\sim 4 \times 10^5$ iPSC-RPE, so this scale is appropriate to provide sufficient cells for repeated drug screening and any subsequent downstream analysis for each donor.

Combining the ability to maintain undifferentiated hiPSC cultures with efficient iPSC differentiation that can be carried out in a convenient, cost-effective adherent plate format, without the need for extensive adjustments between different donor lines, provides the basis for a scalable process to generate hiPSC-RPE from large patient populations. In contrast to the bulk expansion of cell numbers that are necessary to build large banks of allogeneic cell products for potential therapeutic applications, our population-based drug screening protocol uses donor-specific RPE and requires expanding the scale of the number of individual lines that can be processed efficiently and cost-effectively. The plate format we have adopted reduces the cost of culture and differentiation reagents while allowing the expansion of sufficient cells for the drug screening process. We are now employing our workflows to generate RPE from a large bank of iPSC lines we have generated from patients and donors with and those without dry AMD.

The ability to develop and demonstrate the utility of protocols for hiPSC culture and differentiation on commercially available automated workstations without having to design or modify lab-specific equipment has enabled the introduction of automation to a complex experimental process in our academic laboratory. We are able to thaw multiple iPSC lines simultaneously and maintain them in undifferentiated culture, allowing seeding for simultaneous RPE differentiation, without manual handling. Currently, RPE expansion and maintenance are conducted manually due to space limitations, but the recurring tasks of plating the iPSC-RPE lines in multwell plates and culturing them with timed exposure to drugs prior to Seahorse analysis, requiring repetitive accuracy and reproducibility, are conducted with automation. We have worked to design and test these automated protocols to replace manual methods for discrete parts of the iPSC maintenance, RPE differentiation, and testing workflow. Linking these parts together to generate an end-to-end automated process still requires further development of scheduling software, which is currently underway. Further information of our experiences with the workstations, together with details of the control scripts, is available on request from the authors.

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