Automated in-process characterization and selection of cell-clones for quality and efficient cell manufacturing

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Abstract Delivery of safe, effective and reliable cellular therapies, whether based on mesenchymal stromal cells (MSCs) or induced pluripotent stem cells (iPSCs), demand standardization of cell culture protocols. There is a need to develop automation platform that enables the users to generate culture expanded human cell populations that improves the quality and reduces batch-to-batch variation with respect to biological potential. Cell X^{TM} robot was designed to address these current challenges in the cell fabrication industry. It utilizes non-invasive large field of view quantitative image analysis to guide an automated process of targeted “biopsy” (cells or media), “picking” (selection) of desired cells or colonies, or

“weeding” (removal) of undesired cells, thus providing an unprecedented ability to acquire quantitative measurement in a complex heterogeneous cell environment “in process” and then to act on those measurements to define highly reproducible methods for cell and colony “management” based on application specific critical quality attributes to improve the quality of the manufactured cell lines and cell products.

Keywords Automation · Stem-cell manufacturing · Cell-picking · Imaging · Performance-based cell selection · Stem cells

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Introduction

Both, induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) represent important cell resources for cell-based therapies, disease modeling and pharmaceutical applications (Takahashi et al. 2007; Ng et al. 2017; Ramkumar et al. 2017; Stoltz et al. 2015; Piuze et al. 2018; Piuze et al. 2017; Mantripragada et al. 2019; Mantripragada et al. 2018a, b). There are currently over 800 ongoing clinical trials using MSCs as per clinicaltrials.gov records, and approximately 1900 hPSC lines, registered in hPSC registry (hPSCreg) (Kurtz et al. 2018; Selmann et al. 2016). However, despite the enormous progress and promise, several unresolved challenges

continue to plague the MSCs and iPSCs field including: (1) highly subjective manual decision-making steps that are ubiquitous in current cell biology and cell processing methods (Chen et al. 2014; Cerbini et al. 2015), (2) a lack of standardized methodology between laboratories that likely introduces more variability (Mantripragada et al. 2019), (3) the variation in the quality of these cells within and between individuals (Mantripragada et al. 2018a, b; Wrighton 2017; Qadan et al. 2018; Haasters et al. 2009; Russell et al. 2010; Vitale et al. 2012); and (4) clonal differences among cell populations. In the iPSCs world, labs invest great energy in making subjective value judgments regarding the “quality” of individual clones and “completeness of reprogramming”.

Precision medicine, which aims to treat diseases with special consideration for individual’s biological variability, has been publicized to be one of the paramount pillars of the future healthcare, and is projected to attain a \$96 billion market in the coming 7 years (2017–2024) (Kutepatil 2017). Such clinical diagnostics and therapies demand unprecedented rigor in characterization, control, documentation and reproducibility.

Judgments are often based on the appearance of iPSC colonies (e.g. size, shape, morphology, density, texture, border). Skilled technicians manually “pick” the best colonies, most often with little or no documentation of the initial colony appearance. During the iPSC expansion, subjective decisions are made continuously regarding the differentiation status of the cells under expansion. Technicians spend hours deciding when and how to passage iPSC cells, based on their judgment/experience, seeking to avoid over confluence that may increase differentiation, and identifying and removing cells that show evidence of unwanted differentiation (“weeding”) (Wrighton 2017; Schwarz et al. 2018).

In the MSCs world, researchers and manufacturers use a wide range of protocols to obtain their MSC-based products, which has prominent impact on the characteristics of the product. In addition, there is still a need to understand the composition, phenotype, and biological activity of the MSCs present in a given tissue and donor (Mendicino et al. 2014; Galipeau and Sensébé 2018).

There is a need for appropriate tools and techniques to understand and study biological variability and standardize cell culture related protocols to minimize

experiment-to-experiment variation, and lab-to-lab variation, and ultimately meet the regulatory requirements for defined cell-culture conditions and better cell characterization for clinical application of these cells. One way to approach this problem is through high-content image analysis grounded on defined and measurable critical quality attributes (CQAs) in the adult stem cell and progenitor populations and induced pluripotent stem cells (iPSC). These attributes must be defined based on quantitative and standardized metrics. Based on this information we can then act systematically, and reproducibly using appropriate tools, to rapidly identify and isolate desired cells or clones or to delete undesired cells/clones, for quality-controlled, documented, and cost-effective cellular therapies (Fig. 1).

To address these challenges, we have developed an integrated automated Cell X^{TM} platform and Colonyze^{TM} (Fig. 2). Cell X^{TM} platform is a robotic system of precision movement and fluidics that is integrated with an automated inverted fluorescent microscope. This enables tracking of live cells growing in the culture dish by rapid capture of high resolution large field of view (LFOV) (“Google Earth”) images, automated image analysis, and then precision manipulation of live cells based on the image analysis (biopsy, selection or removal of cells). Cell X^{TM} platform can also efficiently perform standardized media changes (Fig. 2a). Colonyze^{TM} is a generalizable automated multimodal LFOV Image Analysis software platform. Colonyze^{TM} analysis is based on ASTM standard method F-2944-12 (ASTM 2944). This allows standardized quantitative characterization of the physical and biological attributes of all cells, colony-forming units and their respective colonies in a LFOV image. These attributed can then be used to define the CQAs for automated manipulation of cells (Fig. 2c). Cell X^{TM} also precisely documents the workflow including: (1) location of the cells and colonies in the LFOV; (2) physical and biological attributes of the cells and colonies manipulated; and (3) the process parameters used during the workflow for quality control, which allows repeatability and reproducibility for all experiments within and between laboratories.

We believe that these combined attributed in the Cell X^{TM} Platform (including Colonyze^{TM}) addresses the current limitation in the manual methods by: (1) standardized quantitative metrics for cell and colony selection derived from live-cell imaging; (2)

Fig. 1 Traditional method of MSC expansion (top-row) vs critical quality attributes (CQA)-based selection (bottom-row) in a systematic, standardized, reliable and reproducible fashion using Cell X^{TM}

systematic, repeatable, reproducible, and documented manipulation of cells during expansion; (3) development of standardized image-based profiles for a range of cell types based on ASTM standards for use as CQA-driven process controls and release criteria; and (4) design of rapid scalable automated processes with defined validation histories for cell Banks, CLIA certified laboratories, and automated GMP fabrication facilities.

Materials and methods

Automated LFOV cell & colony analysis—Colonyze^{TM}

The Cell X^{TM} Platform integrating Colonyze^{TM} 4.0 is a user-friendly Windows^{TM}-based automated quantitative cell and colony analysis application. It was written in 64-bit Visual Studio^{TM} C/C++ with a graphical interface built in Qt^{TM} 5.7. It makes use of CUDA^{TM}/GPU programming to accelerate computationally intensive algorithms and OpenGL^{TM} for 2D and 3D graphics visualization. Colonyze^{TM} provides workflows for quantitative analysis of brightfield, phase contrast, 2D and 3D fluorescence images, as well as time-lapse videomicroscopy images acquired using any microscope system (i.e., Leica, Olympus) (Fig. 2a). Quantitative image analysis can be performed on individual images or can be batch-processed, with final data exported to spreadsheet. It also includes an interactive interface for post-processing visualization and revision. A variety of stem cell populations have been analyzed using this software

including hematopoietic, bone marrow, fat, synovium, periosteum, bone and cartilage, as well as reprogrammed iPS cells from fibroblast and peripheral blood cells. These quantitative methods have been adopted in accordance with principles articulated as an ASTM Standard Method for “Automated Colony Forming Unit (Assays-Image Acquisition and Analysis Method for Enumerating/Characterizing Cells and Colonies in Culture—ASTM # F2944-12). Using this quantitative, standardizable, and reproducible automated tool will help overcome limitations associated with manual methods for cell processing including cell and colony counting as well as colony selection, which are extremely time-consuming, inaccurate, and inconsistent within and between users (Powell et al. 2016, 2007).

The quantitative metrics available to the user for any region of interest (including whole wells) include, but are not limited to: cell number, cell morphology metrics, confluence, colony number, individual colony level attributes (cell number, density, population morphology metrics), and multimodal attributes at cell and colony level (e.g. area and intensity of fluorescence, and overlay of multiple channels of fluorescence) (Fig. 2c).

Automated cell and colony precision manipulation—Cell X^{TM}

The Cell X^{TM} device integrates imaging, cell-manipulation, fluid handling, and motion control systems. The imaging system currently comprises of an Olympus one-deck IX83 inverted epifluorescent microscope (Olympus Corp., Tokyo, Japan), Retiga R3

Fig. 2 **a** Cell X^{TM} automated imaging and cell manipulation system. The robotic platform comprises of eight major components: (1) Automated microscope, (2) Monochrome CCD camera, (3) Submicron precision stage, (4) Two slots for standard cell culture plates (manual or robotic loading capabilities), (5) Four microfluidic pumps, (6) Microliter precision syringe pump, (7) LCR positioner carrier for disposable tip rack, and (8) Quantitative large field of view image analysis using Colonyze^{TM}. **b** Cell X^{TM} device occupies approximately 31x22x27 in³ (lwxh) and is positioned in Biospherix Xvivo System for its operation in cell-culture compatible environments. **c** Using Colonyze^{TM} automated cell

analysis software, we can process large field of view (LFOV) fixed (i, ii) and live cell cultures (phase contrast-PC -iii and fluorescence-FL images-iv) of various cell types including adult stem cells (iii, iv) and iPS cells (v,vi) to obtain critical quality attributes of desired cell populations. Quantitative analysis of cells and colonies in 3D hydrogels (2D slice in the back and 3D reconstructed view of the cartilage-derived colonies in alginate hydrogel in the front—vii) and dynamic metrics assessment of colony formation from live phase contrast time-lapse images (mitotic events in time series—grey level = time frame, yellow arrow = founding cell, red outline = colony footprint—viii) can also be performed

monochrome CCD camera (Q Imaging, Surrey, British Columbia) and Lumencor Sola SE 365 (Lumencor Inc., OR, USA) light engine, which together were used for LFOV image acquisition.

The apparatus for cell-manipulation system comprised of a robotic microfluidic syringe, currently the Smart Syringe^{TM} (Parker Hannifin, OH, USA) with a dynamic range from 1 to 1000 μl . An automated sterile tip rack enabled exchange of sterile filtered disposable

tips (Thermo Fisher Scientific, MA, USA). The fluid handling system for media removal and replacement comprised of four peristaltic pumps (model: WPX1-P1/8FA4-WT6-BP, WELCO Co., Tokyo, Japan). The motion control system for plate and fluidic tip positioning comprised of eight 2-D axis developed by Parker Hannifin. These enable precise and reproducible motion for imaging, biopsy, picking, weeding,

and media changes. Cell X^{TM} software integrates all the components and functions.

A Python based software platform communicates with the individual components using C# and C++ servers. A user-friendly, intuitive, Cell X^{TM} graphical user-interface (GUI) allows the user to plan and execute imaging, image analysis, biopsy, picking, weeding or media change actions individually or as a planned series of steps that comprise defined protocols. Task lists can be generated or downloaded to enable batch processing of a series of wells or plates using a single protocol.

A Biospherix Xvivo^{TM} system was designed for the Cell X^{TM} device (Fig. 2b). This provides a fully enclosed HEPA filtered laminar flow environment with independent controls of a workspace enclosing the Cell X^{TM} device, a work space level buffered pass through chamber, and an upper chamber for placement of media. This upper chamber enabled media to be changed without opening the workspace, minimizing materials traffic. Operating conditions in the work space can be selected in the respective ranges: O₂: 0–25%; CO₂: 0–20%; relative humidity: 0–85%; temperature: 0–45 °C; chamber pressure: 0–100 Pa; particles (> 0.05 µm): 0–1000/m³. The Cell X^{TM} hardware and software are used in ongoing development for application-specific enhancements that improve the speed, analytical specificity, and performance (Kwee et al. 2017). A general work-flow of operations using Cell X system is shown in Fig. 3a, the systematic documentation of the process and output files is shown in Fig. 3b that validates the effectiveness of the overall system and the general in-process image processing steps along with the variety of image processing algorithms implemented are shown in Fig. 3c. The choice of the processing steps and algorithms used can depend on the image types (phase, fluorescence, brightfield) and cell types in culture.

Imaging System

Automated IX 83 microscope provided multimodal image acquisition, including brightfield, phase contrast and fluorescence images. Four objectives were used (2×, 4×, 10×, and 20×), though the motorized turret accepts up to six objectives. Up to eight excitation/emission filter cubes can be accommodated on the IX 83. A fluorescence illuminator (Sola SE) connects via a 3 mm diameter liquid light guide to the

microscope, and generates white light (wavelength range: 350–680 nm) by combining the outputs of five solid state light sources. This provides a longer working lifetime and mercury-free source. Automated control of light intensity is available for imaging of photosensitive live specimens. Retiga R3 14-bit, sensor-cooling camera with a charged coupled device (CCD) array of 1920 × 1460, and pixel size of 4.54 µm × 4.54 µm is C-mounted onto the microscope for image acquisition and storage. Binning modes of 1 × 1, 2 × 2, 4 × 4, 6 × 6 and 8 × 8 are supported. For bin factor of 1, the pixel resolution of the images acquired at 2 × is 2.27 µm/pixel, 4× is 1.135 µm/pixel, 10× is 0.454 µm/pixel and 20× is 0.227 µm/pixel.

Cell X^{TM} device can accommodate any standard foot print microtiter culture plate (i.e. 128 × 85 × 23 mm). These include 1-well, 4-well, 6-well, 12-well, 24-well, 48-well, and 96-well configurations or higher. Vendor-specific variation in well positioning was required, and stored for general application. After the scan, all the individual images were stitched in a computationally efficient manner to create a unified montage LFOV of any region of interest, most often whole wells. The LFOV montaged image, along with the imaging-parameters are saved in the directory. The montaged image is immediately available to the user for inspection. The user has the option of manually planning biopsy, picking, weeding, or media change options, or selection of additional imaging options.

Cell manipulation system

The Smart Syringe^{TM} is a light-weight, compact microfluidic syringe pump. A servo motor with high resolution encoder enabled low volume aspirations/dispense of fluids (range: 10–1000 µl for 1000 µl syringe barrel) and low flow performance (range of flow-rates: 150 nl/s to 500 µl/s for 1000 µl syringe barrel) settings. The accuracy and precision of Smart Syringe pump (SSP) is ± 1% and ± 0.1% at 100% full stroke volumes. Various standard 30 mm XP type syringe barrel volumes can be supported on the SSP, ranging from 50 to 1000 µl. A metallic luer on the SSP enabled exchangeable press-fitting of sterile filtered disposable tips of varying volume capacity (250 µl–1000 µl) for fluid or cell biopsies, and picking and weeding functions. The SSP z-axis mount was fitted with a force sensor (LCM201, Omega Engineering).

◀**Fig. 3** Demonstrate the general workflow of operations and processes on Cell X^{TM} device. **a** Illustrates the workflow of cell culture plates through the Cell X^{TM} system. After processing of the tissue source and plating cells in the tissue-culture plates (6,12, 24, 48, 96 well plates or 10 cm dish) in the laminar flow hood under sterile conditions (processes indicated in orange boxes), the plates can undergo any one of the processes on Cell X^{TM} (processes indicated in yellow) and finally be placed in cell culture incubator (process indicated in blue box) until ready for the next steps. **b** Illustrates the Cell X^{TM} output files and formats that are documented systematically for each of the process (Media change, Imaging, In-process image analysis and cell/colony manipulation) to ensure effective conduct of reliable and reproducible research. **c** Illustrates the general workflow for in-process image processing and the various algorithms used for each of the steps to identify cell populations of interest for further manipulation (picking/weeding). (Color figure online)

This sensor provides feedback to the motion control system. This feedback is necessary to properly secure the tip in the metallic luer. It was also used to measure the height of the cell surface to enable precise localization of the tip above targeted cells for effective biopsy, picking and weeding functions, without direct plate contact. Opaque disposable tips enable micron-level precise calibration of the tip position in the x- and y-direction using two optical sensors placed on a printed circuit board. A LCR22 linear positioner carries the disposable filtered-tip rack (Thermo Fisher Scientific, Waltham, MA).

Fluid handling system

Four compact, high performance peristaltic pumps from Welco (Tokyo, Japan) have been installed on Cell X^{TM}. The pumps can meet a wide range of flow-rates (20–900 µl/s) for cell culture media change application. Of the four pumps, one pump is used to aspirate media (aspirate pump) and other three pumps are used to dispense different fluid media (dispense pumps). Similar to the SSP (z-axis), the aspiration tip is press-fitted to sterile non-filtered disposable tips for media change functions. The aspiration zz-axis mount is fitted with a force sensor (LCM201, Omega Engineering) to provide a feedback to the motion control system. This feedback is necessary to properly secure the tip in the metallic luer and also used to measure the height off the bottom of the cell culture plates to enable calculations for complete, half or third media changes. The use of disposable tips helps

eliminate risks of cross contamination between samples. Dispense pumps connect via three different tubing to the zzz-axis fitted with three metallic autoclavable tips (316 SS) which operate without direct contact with media. These metallic tips can also be sterilized using isopropyl alcohol or hydrogen peroxide to avoid cross-contamination during media change operations. The pumps are connected to PharMed® BPT tubing and can be sterilized either by autoclave or ethylene oxide sterilization fumigation. The other end of the aspiration tubing is connected to an empty bottle to collect used media, whereas the ends of the dispense fluid lines can be connected to a maximum of three different media bags/bottles being used in an experiment.

Motion-control system

Two ACR9000 controllers (Parker Hannifin, Cleveland, OH) were used to integrate the Cell X^{TM} motion. One ACR9000 controls the eight axes (servo and stepper controls) used for image acquisition, cell manipulation, and media change axes (zz, zzz). Of the eight axes, three axes (x,y, xx) are controlled by linear servomotors, which are coupled to sensors for position feedback, thus providing micron level precision and accuracy of location, velocity and acceleration. Five axes (z, yy, zz, zzz, SH) are controlled by high performance stepper motors. The second ACR9000 controller controls the four media pumps.

Application of Cell X^{TM}

In order to demonstrate a representative cell-culture application for Cell X^{TM}:

- 1 Cartilage was obtained from lateral femoral condyle of total knee arthroplasty patients. Tissue was enzymatically digested to isolate cartilage-derived cells. Cells were placed into culture with media changes every other day. At day 10, live-cell phase contrast images were acquired using a 10x objective. LFOV image was processed using Cell X integrated Colonyze. Based on the quantitative image analysis output, individual colonies were picked and transferred to recipient wells for culture expansion to demonstrate viability and importance of performance-based selection. Before-pick and after-pick images were compared

to quantify the efficiency of picking. Ten-days post-picking images were acquired to quantify the differences in the colony performance.

- 2 hiPS cell line DF6-9-9T (WiCell, Madison, WI) was cultured as per supplier protocols in E8 media. Before-pick and after-pick images were compared to quantify the efficiency of picking. Cells were imaged daily after replating to demonstrate viability, preservation of the iPS morphology, and to characterize the cell and colony metrics.

Statistical analysis

The outcome variables measured included (1) time for media change in the different cell culture plates and (2) time for image acquisition of complete cell culture plates at different magnifications and acquisition modes. Mean along with their standard deviations are reported for all testing conducted ($n = 20$).

Results and discussion

Using the Cell X^{TM} graphical user-interface, imaging, media changes and automated image processing were performed as illustrated in Fig. 4a, b. Picking operations were effective, and transplanted cells continued to proliferate effectively with morphology that is unchanged from that of the original colony.

Media change function of the GUI utilizes a “swirl” option to mobilize non-adherent cells and debris prior to aspiration. This moves non-adherent cells and debris to the center of the well for aspiration. Additional wash steps can be performed with appropriate solutions (e.g. complete media or phosphate buffered saline), before replating the well with appropriate fresh media. The GUI allows the user to select the flow rates for aspirating and dispensing fluids during the media change, and the volume of media that needs to be replaced. The location for adding media can be selected, but defaults to the center of each well.

The media change times for different well plates using Cell X^{TM} are shown in Table 1. Note that the process modeled in Table 1a treats each well as a separate item and provides a “swirling” step before aspiration and replacement of in each well. Elimination the swirl step in 24 and 96 well plate reduces media change time to 12 and 32 min per plate,

respectively. Automated media changes are more repeatable and reproducible than manual media changes in all cases and minimizes the contamination problems associated with manual media changes. Moreover, changing media using Cell X^{TM} frees the user to perform other tasks while media change is in process. The user can also program Cell X^{TM} to capture images to document the condition of cells in each well as part of the process, saving the time needed for transfer of plates to another location. Imaging at the time of media change also enables automation of the quantification of confluence and decision-making regarding the timing of passage steps. Overall, the integration of automated imaging and media changes enables “in-process” documentation of all media change steps and automated assay of cell performance (proliferation) between media changes.

Imaging function of the GUI allows selection of individual wells in the plate that need to be scanned. User can select the mode of acquisition (brightfield, phase contrast, fluorescence), magnification (2×, 4×, 10×, 20×), camera gain and exposure settings, bin factor, area to be scanned (complete well area, half-well area and third-well area), stage speed during the scan (lower speeds for live-cell imaging with media in the wells, and higher speeds for fixed/fluorescence cell imaging). Based on the total wells selected for imaging, they can be imaged at different focus plane levels determined by autofocus. Once scanning is complete, montage of the LFOV is displayed in the GUI for further action. The scan times for “Google Earth” imaging all wells in entire 6, 12, 24, and 96 well plates are shown in Table 2. Note, of course, that in many settings it is not necessary to scan an entire well to assay the performance in that well (e.g. confluence). Depending on the application, selection of an appropriately reduced region of interest can speed processing.

Automated image processing modules in the Cell X^{TM} GUI enables users to assess the images created using application-specific profiles (e.g. iPS-reprogramming, iPS-expansion, bone marrow-derived stromal colonies, MSC expansion, ...). Each processed image will display output metrics (colony identification, colony area, colony density, the x,y,z-centroid for each colony, cells/colony, % marker expression area within the colony), as well as highlight the periphery of individual colonies or cell boundaries in the montage image, as shown in Fig. 2c. Based on these

metrics, one can quantitatively define the cells or colonies of interest and selectively pick and transfer the cells for future experiments. It takes 10 s to process

image of around 2 GB. The iPS expansion module includes automated detection of regions of differentiation. This can be used to objectively compare the

Fig. 4 Demonstration of some of Cell X^{TM} applications: **a** Large field of view (LFOV) live-phase contrast image was acquired of colonies derived from human cartilage after 10 day primary culture in a 6-well plate using Cell X^{TM}. Based on the quantitative outcomes reported by Colonyze^{TM}, two morphologically distinct colonies (A1-1: yellow box and A2-1: green box) were selected and transferred to two individual wells of a 24-well plate. Pre- and post-pick images and the pick efficiency of the colonies selected were stored for future reference (A1-1 and A1-2; A2-1 and A2-2). LFOV images of the 24-well plates wells were acquired 10 days post-picking of the two individual colonies to determine cell survival, proliferation and differentiation potential (A1-3, A2-3). **b** iPSC colony picking using Cell X^{TM} iPSC colonies are more readily picked than more adherent fibroblastic cells. iPSC clones of interest can be identified based on morphological assessment (based on phase contrast images) or cell-surface marker assessment (based on TRA1-60 positive staining determined using fluorescence images). Positively stained cells can be precisely picked for further expansion leaving behind unstained (not fully or successfully reprogrammed) iPS cells. **c** iPS-derived cardiomyocytes can be selectively isolated based on rhythmic beating patterns or cell-surface marker staining or cell morphology for expansion or assays to obtain desired mature cardiomyocyte population. (Color figure online)

performance of iPS clones and lines, and/or to guide “picking” and “weeding” interventions.

The “pick/weed” function in the GUI allows the user to selectively biopsy (remove for assay), pick (select and replat), or weed (remove and discard) the selected cells or colonies. Selection of the colonies of interest can be performed manually or based on automated image analysis. For manual selection of the colonies, pick locations can be added by clicking on the montage and adding them to the “picking task list”. Aspiration and dispense flow-rates and

aspiration heights can be adjusted based on the cells being used (the cell adhesion to the cell-culture plate differs between cell populations). The cells collected at biopsy/pick/weed points are directed to user selected sites in the recipient-plate. The “weed” samples are generally directed to the Cell X^{TM} disposal site. Once images and task lists have been created, pick/weed operations can occur rapidly and depending on the actions requested, the rate of one pick can range between 40 and 90 s. If an application requires that the picking tool (needle tip) needs to be changed between samples, each tip change requires an additional 115 s. Cell X^{TM} also captures the pre- and post-pick image of each pick location to enable assessment of picking efficacy and documentation and adds additional media to the pick-well to compensate for the media being aspirated and adds media to recipient-plate to make up to the working volume.

The precision and biological integrity of the Cell X^{TM} biopsy/pick/week operations represents a significant improvement over previous devices which pick cells from 2D surfaces by scrapping them off of a surface (Haupt et al. 2009; Zoldan et al. 2010). The Cell X^{TM} method of pulling cells off of the surface using shear force, prevents crushing or smearing of cells, which may compromise cell yield, cell viability, and the composition of bioactive factor in the sample after replating. Key elements in the Cell X^{TM} process include calibrating location of each disposable tip press-fitted on the luer attached to SSP by optical sensor. We have found any time, the a disposable tip is press-fitted onto a luer, the position of the tip in the (x,y) space will vary by ($\pm 105 \mu\text{m}$, $\pm 170 \mu\text{m}$)

Table 1 Complete media change times (mean $\pm$ SD) for different cell culture plates from the time the plate is placed to the time it is ready to be removed from the device

Cell culture plate	Well working volume (ml)	Media change time for 1 well (min)	Media change time for complete plate (min)
96-well plate	0.15	0.43 ± 0.002	41 ± 0.4
24-well plate	0.5	0.67 ± 0.0022	16 ± 0.08
12-well plate	1	1 ± 0.003	12 ± 0.062
6-well plate	2	1.8 ± 0.005	11 ± 0.06

The media change time includes a swirl motion to collect the debris at the center of the well ($\sim 5\%$ of total time), aspirating and dispensing the working media volume in the well at setting of $120 \mu\text{l/s}$ and $500 \mu\text{l/s}^2$ ($\sim 55\%$ of total time) and axis motion to switch between aspiration and dispense tip ($\sim 40\%$ of total time). Note: To reduce total media change time, swirl is an optional step and higher pump speeds can be used to aspirate and dispense depending on cells in culture. Eliminating the swirl step in 24 and 96 well plate reduces media change time to $12 (\pm 0.06)$ and $32 (\pm 0.31)$ minutes for complete plate, respectively

Table 2 Image acquisition time for entire wells on entire plates varies with the magnification used to collect the image

Cell culture plate	Fields of view per well			Cell culture condition/acquisition mode	Scan time per well/scan time for complete plate (min)		
	10×	4×	2×		10×	4×	2×
96-well	99	30	1	Fixed cells or Live FL mode	0.6 ± 0.001/56 ± 0.08	0.2 ± 0.0003/23 ± 0.05	0.007/0.7 ± 0.002
				Live PC mode	0.9 ± 0.002/90 ± 0.18	0.4 ± 0.001/36 ± 0.06	0.01/1.1 ± 0.003
24-well	266	63	12	Fixed cells or Live FL mode	1.6 ± 0.005/37 ± 0.08	0.5 ± 0.001/12 ± 0.04	0.09 ± 0.0001/2.1 ± 0.009
				Live PC mode	2.5 ± 0.01/60.1 ± 0.33	0.8 ± 0.002/19 ± 0.08	0.14 ± 0.0005/3.4 ± 0.015
12-well	540	120	20	Fixed cells or Live FL mode	3.2 ± 0.02/38 ± 0.07	0.9 ± 0.002/11 ± 0.07	0.14 ± 0.0006/1.75 ± 0.006
				Live PC mode	4.9 ± 0.04/59 ± 0.5	1.5 ± 0.009/18 ± 0.07	0.24 ± 0.0012/2.9 ± 0.02
6-well	1240	238	48	Fixed cells or Live FL mode	7.6 ± 0.06/46 ± 0.4	1.8 ± 0.01/11 ± 0.06	0.35 ± 0.0018/2.1 ± 0.018
				Live PC mode	17.6 ± 0.07/105 ± 0.46	3.9 ± 0.03/24 ± 0.03	0.6 ± 0.002/3.4 ± 0.02

Scan time (mean ± SD, n = 20) for individual wells and entire plate are summarized for 2×, 4× and 10× images below. (FL fluorescence, PC phase contrast)

(n = 100). Thus, this difference in tip location must be accounted for during pick/weed/biopsy process.

Cell imaging and manipulation validation

Figure 4a demonstrates successful picking of two morphologically distinct clonal chondrogenic progenitors-derived colonies present in a 6-well plate, and transfer of viable cells to a 24-well plate, where their performance and morphology continue to reflect the attributes of the founding colony. (Figure 1) Picking to separate colonies of different biological potential has substantial benefit of traditional methods in which all of the cells in Fig. 3-1a would be passaged together, co-mingling the progeny of the many colonies present in a “competitive selection” process, where only the most rapidly proliferating colony or colonies would survive. In this process, the user fails to select that actual clone of cells or type of cells that contribute to their final population, becoming a passive recipient of the outcome of a process that is largely determined by the founding cells that happened to be present in the

initial plate. “Performance-based selection” that separates cell populations with different attributes, enabled examination of the biological potential of less aggressive cells that would normally be inaccessible in a “competitive expansion” process. Using Colonyze™, the quantitative attributes of any selected cell population can be documented, so that future picking can be repeated in a manner that searches for and selects populations with attributes that have been found to be desirable. This systematic collection of data and archiving of original images enables a “machine learning” approach to discovery and refinement of parameters that can be used to optimize cell and colony selection in future processing and to define critical quality attributes (CQA) at each stage in a cell isolation and expansion process.

Figure 4b demonstrates picking of completely reprogrammed iPS cells colony, as indicated by positive TRA1-60 staining (3B-ii), leaving behind a portion of the colony that did not stain positive for TRA1-60 (3B-iii), as indicated by the white arrow. This illustrates the capacity of the Cell X™ device to

selectively pick the cells of interest, and weed-out the unwanted cells in the primary culture. This illustrates the importance of the versatility that is provided in the Cell X^{TM} hardware and software design, which is enabled in the Cell X^{TM} GUI.

Figure 4c demonstrates the picking of iPS-derived pre-cardiomyocytes from any standard tissue culture plate (donor plate). The region of interest (pre-pick: 3C-i, see supplementary material) can be identified based on rhythmic beating patterns, as demonstrated by the corresponding video (pre-pick video: 3C-i, see supplementary material) of the field of view shown below each image, or based on cell-surface marker staining (not shown here). Once the cells of interest are identified, Cell X^{TM} device can be used to precisely pick these cells (post-pick: 3C-ii, see supplementary material), as well as record the video of the field of view post-picking (post-pick video: 3C-ii, see supplementary material). The cells picked can be placed into any other standard cell culture plate (recipient plate) for further expansion or assessment and can be imaged (day 3 after pick image: 3C-iii, see supplementary material and video: 3C-iii, see supplementary material of a particular cell cluster below).

Cellular therapies and cell-based assays are poised to revolutionize the diagnosis and treatment of serious illnesses. These therapies and assays demand unprecedented rigor in characterization, documentation and reproducibility of the cells being used for treatments. However, current cell culture and therapy approaches defy the intrinsic variability and heterogeneity present in most biological systems. Cell X^{TM} automation device was designed to enable “see, pick, show and know” strategy for cell manipulation, based on quantitative metrics and critical quality attributes (CQAs) of the cells. Cell X^{TM} leverages large field of view imaging, image processing (based on ASTM standards), precision motion, and fluid handling of live-cell culture dishes to define and measure CQAs in stem and progenitor population to rapidly identify and isolate desired cells or clones for clinical diagnostics and safe and effective clinical cell therapies.

Conclusions

Quantitative, standardized, repeatable, and reproducible methods will greatly enable the development and approval of cellular therapies and products based

on robust knowledge of critical quality attributes (CQAs).

The Cell X^{TM} Platform provides an integrated system of automated quantitative cell and colony imaging and analysis as well as tools for precision manipulation (biopsy, picking and weeding) driven by quantitative protocols. This enables the development of fully automated cell processing and fabrication methods to be developed in a manner consistent with the demands of a GMP environment that is entirely free of manual manipulation and subjective decision making. In essence, the Cell X^{TM} platform capabilities enable a user to “See what you have. Pick what you want. Show what you’ve done. Know how to do it again.”

The Cell X^{TM} platform provides the versatility needed for deployment in automated manipulation of a broad range of adult stem and progenitor populations as well as induced pluripotent stem cells (iPS cells). Cell X^{TM} methods have been applied to cells cultured on 2D surfaces as well as 3D hydrogel media. Cell X^{TM} methods can be deployed and used in a setting of exploratory basic and translational research, either in a lab-specific application or in a core facility, but are designed to be scalable to applications in fully automated industrial setting, including GMP manufacturing.

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Compliance with ethical standards

Conflict of interests GFM and KP are named inventors on two issued and one pending patent that have been licensed by Cell X Technologies.

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