Large Field of View Quantitative Phase Imaging of Induced Pluripotent Stem Cells and Optical Pathlength Reference Materials

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ABSTRACT

Induced pluripotent stem cells (iPSCs) are reprogrammed cells that can have heterogeneous biological potential. Quality assurance metrics of reprogrammed iPSCs will be critical to ensure reliable use in cell therapies and personalized diagnostic tests. We present a quantitative phase imaging (QPI) workflow which includes acquisition, processing, and stitching multiple adjacent image tiles across a large field of view (LFOV) of a culture vessel.

Low magnification image tiles (10x) were acquired with a Phasics SID4BIO camera on a Zeiss microscope. iPSC cultures were maintained using a custom stage incubator on an automated stage. We implement an image acquisition strategy that compensates for non-flat illumination wavefronts to enable imaging of an entire well plate, including the meniscus region normally obscured in Zernike phase contrast imaging. Polynomial fitting and background mode correction was implemented to enable comparability and stitching between multiple tiles. LFOV imaging of reference materials indicated that image acquisition and processing strategies did not affect quantitative phase measurements across the LFOV. Analysis of iPSC colony images demonstrated mass doubling time was significantly different than area doubling time.

These measurements were benchmarked with prototype microsphere beads and etched-glass gratings with specified spatial dimensions designed to be QPI reference materials with optical pathlength shifts suitable for cell microscopy.

This QPI workflow and the use of reference materials can provide non-destructive traceable imaging method for novel iPSC heterogeneity characterization.

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Keywords: Stem cells, quantitative phase imaging, reference materials

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1. INTRODUCTION

Robust regenerative medicine therapies require a source of stem and progenitor cells. Induced pluripotent stem cells (iPSCs) are a compelling stem cell population that can meet the needs of regenerative medicine. iPSCs are derived from somatic cells that are genetically reprogrammed back to a pluripotent state. The reprogramming process and maintenance of pluripotency during culture expansion requires a trained technician to distinguish heterogeneity of iPSCs and differentiated cells and select iPSCs based on cell morphology using phase contrast imaging [1, 2]. Traditional Zernike phase contrast is primarily used to characterize and identify desired iPSC colonies based on qualitative information about cellular features. Such characterization is subjective and non-transferable between instruments or laboratories. Additionally, Zernike phase contrast features are only maintained in cell cultures where the media surface is relatively flat. Due to the surface tension of cell culture media in well plates, the meniscus-free region is relatively small compared to the entire well, limiting the effective viewing area using Zernike phase contrast.

Quantitative phase imaging (QPI) microscopy is an imaging modality that is different from conventional Zernike phase and can quantitatively determine the phase change in light through cells [3]. We demonstrate the use of QPI to characterize iPSC colony heterogeneity based on dynamic colony mass and area measurements. QPI measurements can also be made traceable to physical reference materials, enabling calibration and comparability between different instruments and laboratories.

2. METHODOLOGY

We developed a large field of view imaging (LFOV) method that performs tiled image acquisition across well plates and characterize iPSC heterogeneity across multiple colonies (Fig 1). In this study, we used a Phasics SID4BIO camera (Phasics S.A., Saint-Aubin, France) which uses quadriwave lateral shearing to perform QPI imaging [4]. QPI methods require physical flattening of the cell culture media to remove the meniscus effect [5]. We implement a method to correct for the non-flat illumination wavefront created by the meniscus and enable LFOV QPI imaging in regions of a well normally obscured in Zernike phase contrast images. We used reference materials to evaluate quantitative capabilities of the developed image processing and analysis workflow. Polymethymethacrylate (PMMA) beads suspended in mineral oil with known diameters and refractive index were evaluated as a stable reference material that can be used well plates. A custom fused silica phase grating with biologically relevant optical pathlength differences (OPD) that mimic those found with stem cells was also evaluated as a potential reference material with defined OPD. This QPI image acquisition workflow in conjunction with reference materials can overcome limitations of Zernike phase contrast and enable quantitative large field of view imaging of stem cells.

2.1 Quantitative Phase Imaging

Imaging was performed using a Axiovert 200M inverted microscope (Carl Zeiss Microscopy, Thornwood, New York) with a motorized stage and incubator system (Kairos Instruments, Pittsburgh, PA), maintaining the stage samples at 37 $^{\circ}$C and 5 % carbon dioxide. QPI was performed using a Phasics SID4BIO camera with a 485 nm LED transmitted light source (Thorlabs, Newton, NJ). Automated large field of view imaging was performed using a custom acquisition script for Micro-Manager [6].

Using a SID4BIO camera, a reference interferogram image was needed to construct the quantitative phase image [7]. To address this need, two wells in a well plate were used. The first well contained the sample of interest with media and the second well contained only media without sample. Interferogram image tiles were acquired on both wells in a positionally matched manner such that the meniscus on the sample tile were positionally matched with the meniscus on the blank wells. This acquisition method was chosen to compensate for the non-flat illumination created by the meniscus. The signal to noise ratio (SNR) was compared between the meniscus corrected and non-corrected QPI images.

Raw interferogram images were batch processed in MATLAB (MATLAB, Natick, MA) using a software development kit from Phasics to produce the QPI image. Resulting QPI images were segmented for foreground and background for reference beads or cells, as described in sections 2.3 and 2.4. Background was corrected using a third order polynomial fit. For each tile, the mode of the background was subtracted across the tile to enable tile-to-tile comparability. The resulting images were stitched using the Microscopy Image Stitching Tool (MIST) [8].

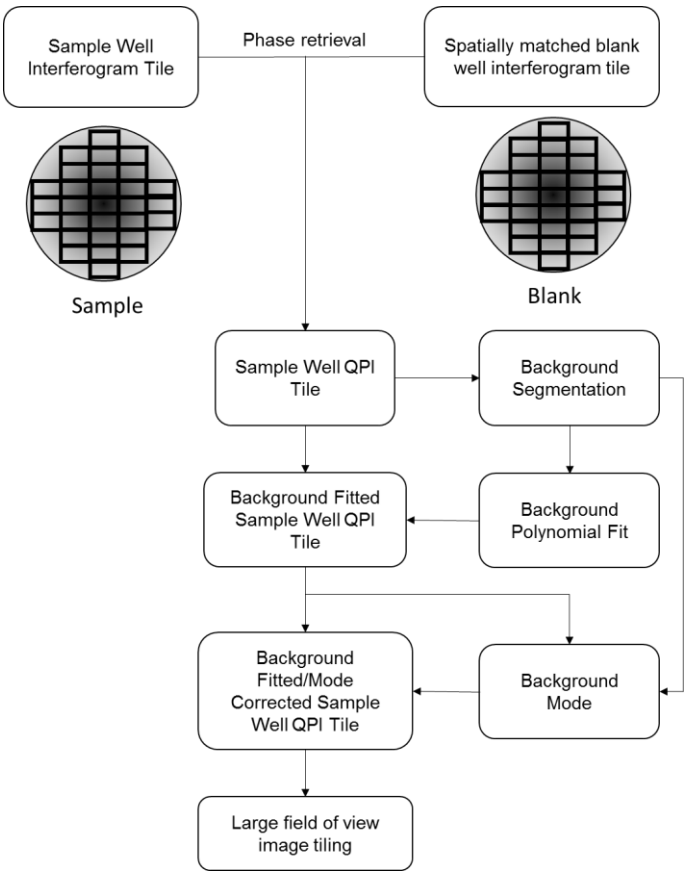

Figure 1: Image acquisition and processing workflow to acquire large field of view tiled QPI image. Diagram of spatially matched acquisition of meniscus in sample and reference sample shown.

2.2 Reference Beads

Polymethylmethacrylate (PMMA) (Cospheric, Santa Barbara, CA) beads were suspended in BioUltra (MilliporeSignma, St. Louis, MO) mineral oil and placed in a 24 well plate. Tiled interferogram and Zernike phase contrast images were taken across a 6x6 tile region (6.2 mm x 4.7 mm) of one well. Reference interferogram images were taken in a well containing only mineral oil. Bead images were processed with and without meniscus correction. Beads were segmented using the active contour method, processed, and stitched with MIST as described in 2.1. Beads were characterized for refractive index and diameter with respect to reference specifications [9, 10].

2.3 Phase Grating

Fused silica phase gratings were fabricated with a period of 40 micrometers (µm), 20 µm feature width, and 100 nanometer (nm) etch depth using reactive ion etching. Samples were imaged using QPI. Resulting images were analyzed in MATLAB. Bilevel waveform pulse analysis was used to characterize optical pathlength differences of the grating when compared to fabrication specifications.

2.4 Cell Culture

ND2.0 iPSCs were plated on Matrigel (Corning, Corning, NY) using E8 Flex culture media (Life Technologies, Carlsbad, CA) in six well plates under three different culture conditions: clump passaged cells plated out of cryopreservation thaw, single cell passaged cells plated out of cryopreservation thaw, and single cell passaged cells at passage three. Cells were cultured in an incubator at 37 ˚C and 5 % carbon dioxide for 24 hours to allow the cells to settle and adhere. After 24 hours, cells were imaged using quantitative phase imaging every hour for two days. Images were processed and stitched as described in subsection 2.1. Image analysis was performed on non-fitted, background

mode corrected images. Colonies were segmented at every time point using the empirical gradient threshold method [11]. Manual tracking was performed to identify clonal colonies that did not merge over the course of the time lapse. Clonal colony time-lapse images were processed in MATLAB to determine the area and mass growth rates of colonies. Optical pathlength difference at each pixel was converted to picograms mass using the constant 0.18 $\mu m^3/pg$ [12, 13]. Paired *t*-test was used to compare mass and area doubling times. Analysis of variance (ANOVA) and Tukey comparison tests were used to compare different culture conditions.

3. RESULTS

3.1 Reference beads

Large field of view images of reference beads were acquired (Fig 2). Zernike phase imaging demonstrated the extent of the meniscus in the well (Fig 1A). Tiles that were not corrected for the meniscus (Fig 2B) had a SNR of 2.52 ± 1.11 (mean ± standard deviation, n=36 tiles). Tiles that were corrected for the meniscus (Fig 2B) had an improved SNR of 13.8 ± 6.53 (n=36) and allowed imaging beads in the meniscus region of the well. Optical pathlength difference measures of the beads reproduced the bead refractive index specifications and dimensions according to reported specifications in both the meniscus-free and meniscus regions of the well (Fig 2D) [9, 10].

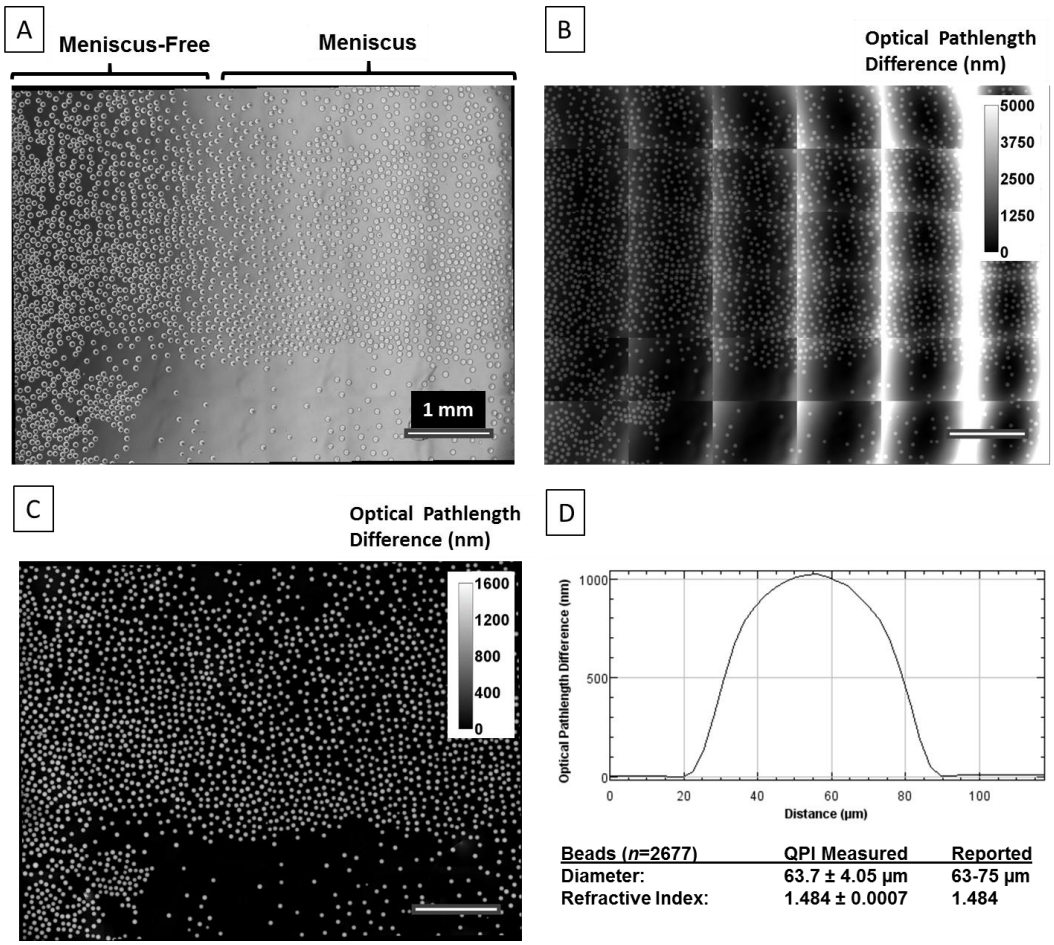

Figure 2: Reference bead characterization across a 6x6 tile region (6.2 mm x 4.7 mm) of a 24 well plate: A) Stitched Zernike phase contrast image of PMMA beads in mineral oil viewing both the meniscus and meniscus-free regions. B) Stitched QPI image of PMMA beads using a single reference image. C) Stitched QPI image using location matched reference images from a blank well to compensate for the cell culture media meniscus. D) Representative line scan of bead from (C). Measured diameter and calculated refractive image of beads from (C).

3.2 Phase grating

QPI images were taken of the phase grating. QPI measurements reproduced optical pathlength difference with 5.6 % error and feature width within 3 % error of fabrication specifications across 27 periods of the grating (Fig 3).

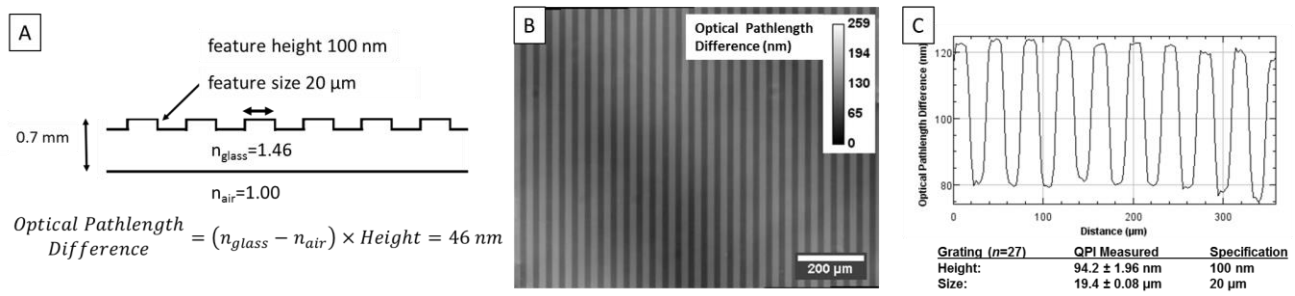

Figure 3: Phase grating characterization: A) Schematic design of phase grating and equation for OPD. B) 10x field of view of phase grating using QPI. C) Line scan of grating. Peak to valley heights and size of spacing were from equation in (A).

3.3 iPSC imaging

LFOV QPI images of iPSC colonies were acquired, processed, and stitched (Fig 4). A portion of the well that was imaged included the meniscus (Fig 4A). Using the developed image processing workflow, the tiled QPI image compensated for the meniscus effect. 70 colonies were identified across all culture conditions. Colonies had a mean mass doubling time of 24.7 hours and a mean area doubling time of 40.6 hours.

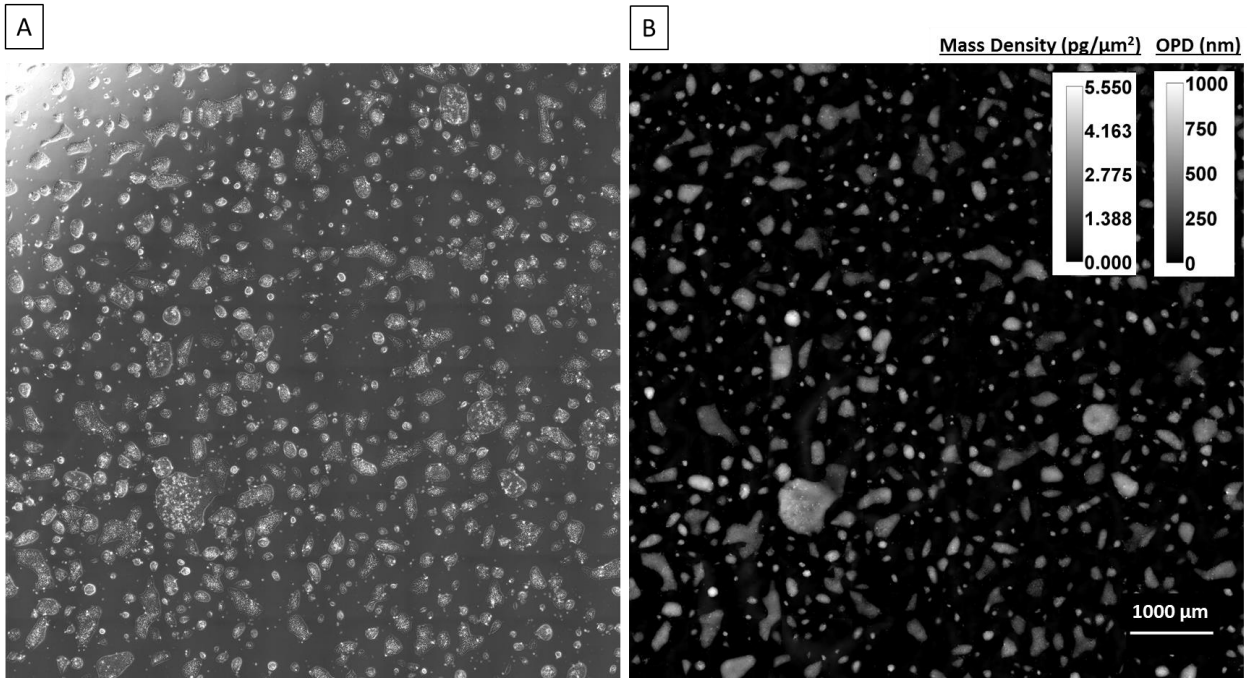

Figure 4: QPI large field of view iPSC colonies of 8x10 tiles (7.4 mm x 7.4 mm). A) Example stitched image of well using Zernike phase contrast image with meniscus. B) Background corrected QPI image with intensity scale expressed in mass density and optical pathlength difference.

Comparing growth rates, mass growth rates were found to be significantly different than area growth rates (Fig 5D). Mass doubling times for iPSCs cultured as single cells were significantly different than clump passaged cells (Fig 5F). No difference in growth rate was found after three passages in the single cell passaged cells. Initial colony mass did not correlate with mass growth rate. Initial colony mass was not significantly associated with mass doubling time.

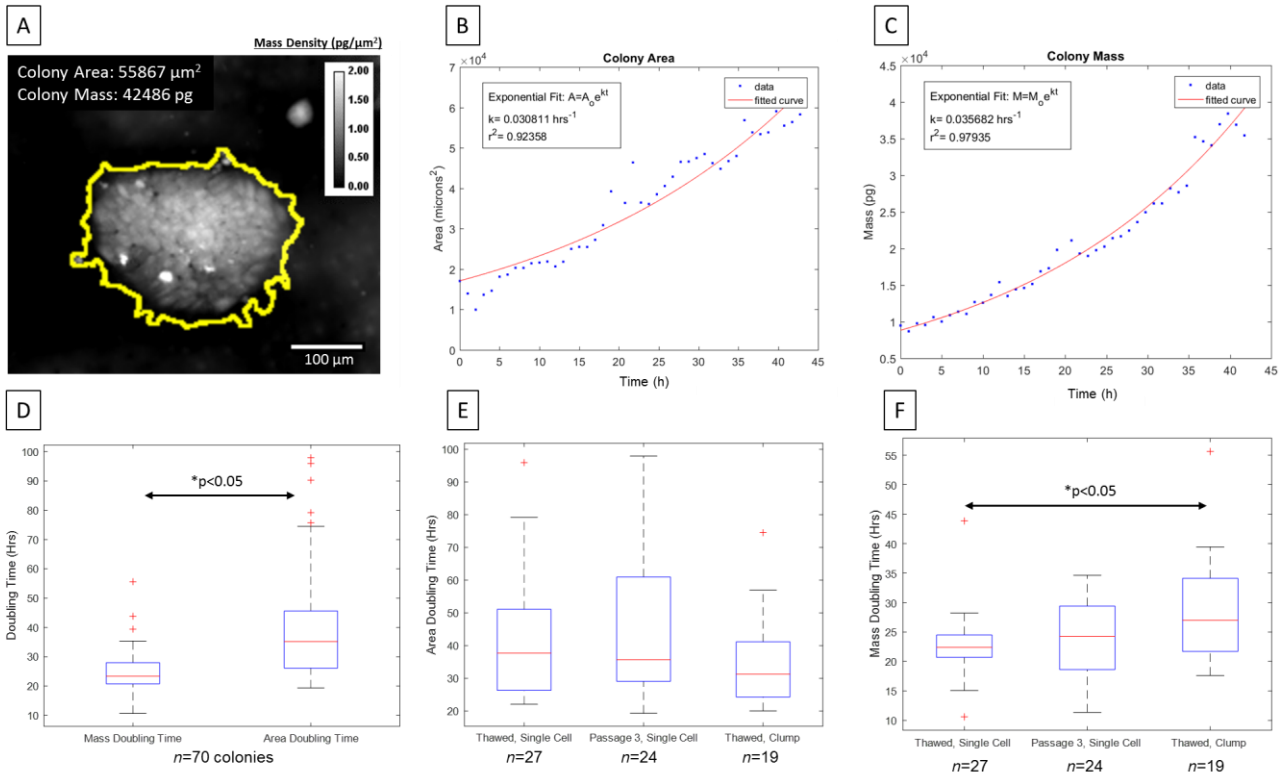

Figure 5: QPI colony analysis. QPI image of representative colony in (A). Based on cell segmentation (yellow), colony area and mass were calculated for each time point. Exponential fits for area (B) and mass (C) was determined for n=70 colonies. Box plots for doubling time based on mass and area for all colonies (D), area doubling time based on culture condition (E), and mass doubling time based on culture condition (F). Box plots in (D),(E), and (F) show the median (red), interquartile range (blue), 1.5 times the interquartile range (dotted), and outliers (red plus).

4. DISCUSSION

We developed an imaging workflow that enables large field of view QPI imaging of cell cultures in well plates with comparability enabled by reference materials. This method overcomes limitations of Zernike phase contrast as a reproducible, quantitative method. This QPI method also enabled imaging in the meniscus to perform imaging of multiple colonies in a well plate to characterize colony heterogeneity. Optical pathlength difference measurements reproduced reference specifications of PMMA beads in mineral oil. The combination of PMMA beads and mineral oil can serve as a stable reference that is not susceptible to evaporative effects and enable long term calibration and traceability of QPI measurements. While useful, the optical pathlength difference created by PMMA beads in mineral oil, approximately 1000 nm, which are higher than the OPD created by biological cells, which are between 50 nm to 250 nm. Additionally, beads may not be the appropriate OPD reference material for all QPI instruments and applications. we found that a custom, fabricated phase grating could be used to produce optical pathlength shifts closer to biological cells. This grating can be used for non-well plate applications and can be placed directly on a QPI instrument without requiring any immersion liquid. A phase grating format also presents the opportunity for an orthogonal measurement of the optical pathlength difference by measuring the diffraction pattern on the back focal plane of a microscope, which is currently

under investigation. Phase gratings with larger etch depths are under evaluation to characterize QPI sensitivity and dynamic range across the biological range of optical pathlength differences induced by cells. Depending on the QPI instrument and application, these materials can serve as a stable reference to calibrate and enable comparability between QPI instruments and experiments.

QPI imaging of iPSC colonies enabled measurement of growth rates by both area and dry mass. Mass growth rates were found to be significantly different than area growth rates. For 2D microscopy techniques like Zernike phase contrast and brightfield, area is the primary measure of cell growth and confluency. QPI provides different metabolic information of the mass growth rate of cells that is not captured by area measures. This could be due to the capability for QPI mass measurements to capture mass changes that occur during the resting or interphase periods of the cell cycle. These periods are not captured by changes in cell area when cells are not actively dividing. Additionally, the contraction and expansion of cell area over the course of the time lapse makes area a poor measure of exponential growth.

Mass growth rate was able to distinguish differences between iPSCs cryopreserved as single cells from those cryopreserved as clump passaged cells. Proliferative ability and self-renewal are critical characteristics of iPSCs. Colonies with the highest mass growth rates may represent the clones with the greatest regenerative capabilities [14]. Further work is needed to confirm the pluripotency state of the cells. The use of QPI to distinguish pluripotency state between stem cells and differentiated cells is under investigation. We expect differences in nucleus condensation and mesenchymal-epithelial transitions as measured by QPI can characterize cell pluripotency state. Beyond culture conditions described here, QPI can enable characterization and identification of other critical manufacturing processes in the culture of iPSCs. This can include assessment of iPSC response to reprogramming methodologies, cell culture media formulations, and differentiation protocols. The quantitative capability and traceability to reference materials of QPI can advance the study and manufacturing of iPSCs as a viable cell therapy for patients.

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