PLURIPOTENT STEM CELLS

Video bioinformatics analysis of human pluripotent stem cell morphology, quality, and cellular dynamics

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Abstract
StemCellQC is a video bioinformatics software tool for the quantitative analysis of human pluripotent stem cell (hPSC) colonies. Our objective was to use StemCellQC to evaluate and compare various experimental culture conditions, cell lines, and treatments and to demonstrate its applicability to PSC problems. Seven key features were identified that provided useful information on PSC morphology, dynamic behavior, and viability. Colony attachment was better on laminin-521 than on Matrigel and Geltrex. Growth rates were similar on each matrix when data were normalized. The brightness/area ratio feature showed greater cell death in colonies grown on Matrigel and Geltrex than on laminin-521 further contributing to an overall greater yield of cells on laminin-521. Four different PSC culture media performed similarly; however, one medium produced batch-to-batch variation in colony morphology and dynamic features. Two embryonic and one induced pluripotent stem cell line showed significant differences in morphology, growth rates, motility, and death rates. Cells from the same vial that became phenotypically different in culture showed measurable differences in morphology, brightness, and motility. Likewise, differentiating and undifferentiated colonies varied in growth rate, intensity, and motility. Three pluripotent cell lines treated with a low concentration of cinnamaldehyde, a chemical used in consumer products, showed adverse effects and differed in their sensitivity to treatment. Our data demonstrate various applications of StemCellQC which could be used in basic and translational research, toxicological and drug testing, and clinical facilities engaged in stem cell therapy.

KEYWORDS
cell death, growth, human embryonic stem cells, live-cell imaging, motility, pluripotent stem cells, undifferentiated cells, video

Significance statement
The authors present various applications of StemCellQC, a label-free video bioinformatics analysis tool for evaluating pluripotent stem cell morphology, quality, and dynamics during in vitro culture. Morphology, growth, motility and death were compared quantitatively using pluripotent stem cells grown on different matrices, in different media, in conditions of stress, and in the

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1 | INTRODUCTION

Human pluripotent stem cells (hPSCs) have the potential to increase our understanding of human prenatal development, identify environmental chemicals and drugs that are toxic to embryos, provide novel models for studying diseases in vitro, and cure a broad spectrum of debilitating diseases. In each application, there is a foundational need for better methods to quantitatively analyze the morphology and dynamic behavior of PSC cultures. In applications of PSC to human therapies, quality control is essential and new methods are needed to ensure that high quality cells are used for differentiation and transfer to patients.

With the introduction of improved systems for live-cell imaging coupled with video bioinformatics software for analysis of time-lapse data, it is feasible to quantitatively study cellular dynamics and noninvasively monitor cell culture quality. Noninvasive imaging has been used to evaluate human embryonic stem cell (hESCs) maintenance, growth, differentiation, and response to experimental treatments. StemCellQC has numerous potential applications in stem cell laboratories that perform live-cell imaging.

The purpose of this study was to quantitatively evaluate and compare the morphology and dynamic behavior of PSC colonies using live-cell imaging data. Analyses were done using StemCellQC in conjunction with various substrates, culture media, cell types, and experimental treatments. Our approach detected significant differences in morphology, growth, motility, death, and survival of PSC in various culture scenarios and provides example applications of StemCellQC to problems in PSC culture and experimentation.

2 | MATERIALS AND METHODS

2.1 | Cells, chemicals, media, and reagents

Three lines of PSC were used: H9 and H1 from WiCell Research Institute (Madison, Wisconsin) and CC3 induced pluripotent stem cells from Dr. Aaron Bowman (Vanderbilt University, Tennessee).

Morphology and dynamic cell processes were evaluated on three substrates: Geltrex (Gibco, Life Technologies, Grand Island, New York), Matrigel hESC—Qualified Matrix (Corning, Life Sciences, Corning, New York), and laminin-521 (BioLamina, Sundbyberg Stockholm, Sweden). Geltrex and Matrigel were diluted with Dulbecco’s modified Eagle’s medium (DMEM, Lonza, Walkerville, Maryland) in a 1:100 and a 1:30 ratio, respectively. Matrigel was prescreened to verify that it supported PSC culture. Laminin-521 was diluted 1:20 with Dulbecco’s phosphate buffered saline with calcium and magnesium (DPBS +, Lonza, Walkersville, Maryland). 6-well plates (Corning, New York) were coated with 1 mL of substrate and left at 4°C overnight then incubated for 30 minutes at 37°C prior to plating cells.

PSC were cultured in mTeSR1, E8, or mTeSR2 medium (STEMCELL Technologies, Vancouver, Canada), which promote self-renewal while maintaining pluripotency. In one experiment, two batches of mTeSR1 were compared. One batch (mTeSR1-A) maintained pluripotency and self-renewal over repeated passages with multiple cell lines. Pluripotency was confirmed by labeling colonies with OCT4 and SSEA3/4 antibodies and by differentiating these colonies into ectoderm, endoderm, and mesoderm (Supplemental Figure 1A,B). The second batch (mTeSR1-B) did not maintain pluripotency and colonies underwent spontaneous differentiation with repeated passaging. Therefore, mTeSR1-A was used for all experiments requiring mTeSR, with the exception of one experiment in which the effects of media were analyzed and the two batches of mTeSR were explicitly compared.

PSC were maintained in laminin-521 coated Falcon 6-well tissue culture plates containing 1.5 mL mTeSR1-A (except in experiments in which E8, mTeSR2, or mTeSR1-B were also used). Plates were incubated with 5% CO2 at 37°C and a relative humidity of 95%. Medium was changed daily, and cells were passaged when they reached approximately 70% confluency. When testing substrates (Matrigel, Geltrex and laminin-521), cells were adapted to their respective substrates for at least three passages prior to experimentation. For passaging, cells were washed with calcium/magnesium free Dulbecco’s phosphate buffered saline (DPBS-, Lonza, Walkersville, Maryland) then treated with Dispase (STEMCELL Technologies, Vancouver, Canada) to remove them from the culture plate. Colonies were lifted during passaging using disposable cell scrapers (Thermo Fisher Scientific, Pittsburgh, Pennsylvania). Colony suspensions were counted and approximately 300 colonies were distributed into a well that had previously been coated with Matrigel, Geltrex, or laminin-521. Additional details on our method of hESC culturing have been published.
FIGURE 1 Analysis of H9 hESC colony morphology, attachment, and growth on Matrigel, Geltrex, and laminin-521 during culture in mTeSR1-A. A, Solidity of hESCs grown on three matrices. * indicates range over which means were significantly different. P values ranged from .05 to .0001. B,C, Colonies with high solidity. D,E, Colonies with low solidity. F, Attachment efficiency of hESCs on Matrigel, Geltrex, and laminin-521. G, Confluency of hESCs on Matrigel, Geltrex, and laminin-521 72 hours after plating. H, Increase in hESCs colony area on Matrigel, Geltrex and laminin-521. For F and G, data are plotted as means ± SEM for three independent experiments. Data in Figure H are means ± SEM of three independent experiments where each experiment had 8-11 colonies/group. Area data (Figure H) were normalized to enable direct comparison of growth on different matrices. All Dunnett’s post hoc tests used Matrigel as a reference group to test for significance. In Figures F and G **P < .01 and ***P < .001. Scale bar = 45 μm
Apoptosis features

Frame 1
(A)

Frame 2
(B)

Frame 3
(C)

Frame 4
(D)

10 hours
(E)

20 hours
(F)

36 hours
(G)

53 hours
(H)

Matrigel

Geltrex

Laminin 521

MitoSox
(O)

Trypan blue
(R)

Figure 2 Legend on next page.
2.3 | Collection of time-lapse data

A BioStation CT high content incubator with a microscope and camera (Nikon, Tokyo, Japan) was used to collect time-lapse images of PSCs, while maintaining appropriate temperature, CO₂ level, and humidity. For each BioStation experiment, PSC colonies of 200-300 cells were passaged into 6-well plates containing mTeSR1 medium. After 24 hours in a standard incubator, the medium was replaced with 5 mL of fresh mTeSR1, and the plate was transferred into the BioStation CT. Three by three tiling was used to capture approximately 10 different areas of interest in each well at 10× magnification every hour for 48-72 hours. Areas were chosen which contained relatively few colonies so that single colonies could be studied throughout incubation. Only colonies that remained in the field during the entire incubation period were used for analysis. A minimum of 10 colonies were used per each experimental condition.

2.4 | Analysis of video data using StemCellQC

StemCellQC is a video bioinformatics software platform that analyzes time-lapse images of hPSC colonies and produces graphs depicting 25 features related to cell morphology and dynamics. StemCellQC was written and developed with the MATLAB 2015a programming environment. The MATLAB stand-alone executable version of this algorithm and test data are available online at http://vislab.ucr.edu/SOFTWARE/software.php. The stand-alone executable requires the installation of the 64-bit version of MATLAB Runtime R2015a (8.5) available at http://www.mathworks.com/products/compiler/mcr/. BioStation images were imported into StemCellQC, and tiled images were stitched together using a patching feature in StemCellQC. Each colony was masked using StemCellQC’s edge-detection based algorithm, and data were extracted. Each experiment was performed three times, and the data for each of the 25 features were averaged and plotted with the SE of the mean (SEM). Graphs were analyzed to identify those features that were different in each experiment. Prior to analysis, area was normalized by setting the first frame to 1. Motility features were normalized by setting the initial point in each video to zero.

2.5 | Statistical analysis

All statistical analyses were done using GraphPad Prism (GraphPad, San Diego, California) or Minitab (State College, Pennsylvania). Attachment efficiency and confluency data (Figure 1F,G) were analyzed using a one-way ANOVA with a Bonferroni post hoc test in GraphPad Prism. All other data were analyzed using a two-way ANOVA in GraphPad Prism when data satisfied the assumptions of ANOVA (normal distribution and similar variance). When data did not satisfy the assumptions of ANOVA, they were transformed using a Box-Cox transformation and subjected to a two-way ANOVA using Minitab. When results were significant for comparison of two groups, the Bonferroni post hoc test was used (Figures S1-N, 6E-H, 7A-F, and Supplement Figures 1 and 2). When differences were found among three groups, post hoc testing was done with Dunnett’s test using a reference group (Figures 1A,L,M, 2S, 3A-F, and 4A,B,I,M,N). Groups were considered significantly different for P ≤ .05.

3 | RESULTS

3.1 | Overview of the study

To evaluate the morphology and dynamics of PSCs in culture and to demonstrate the applicability of StemCellQC to PSC time-lapse data, colonies were examined on different substrates, in different culture media, while undifferentiated or undergoing differentiation, and during treatment with an environmental chemical. In addition, three lines of PSC and a line with an aberrant phenotype were studied. The seven most useful features for comparing groups were: (a) solidity which refers to the number of pixels in the segmented colony divided by the number of pixels in a convex hull fitted around the colony; the closer solidity is to 1, the higher the solidity and the more circular the colony; (b) colony area, which increases with colony growth; (c) number of protrusions, which are small dynamic processes on the colony surface that increase as colonies grow; (d) brightness/area ratio, which is the total brightness of a colony divided by its total area; this feature allows quantification of dying cells which adhere to the surface of colonies and have high intensity; (e) average intensity, which is an indicator of cell death and also reports on spreading, which lowers overall intensity; (f) total distance traveled, which measures how far a colony has moved; (g) total displacement, which measures the distance a colony has moved from its starting position; and (h) perimeter, which measures a continuous line around the edge of a colony and increases as colonies grow.
FIGURE 3  Legend on next page.
3.2 | Effect of the substrate on colony morphology

Solidity is one of several features in StemCellQC that evaluates colony morphology. As shown in the StemCellQC graph of solidity, colonies plated on Matrigel and Geltrex had similar solidity, while those on laminin-521 had significantly lower solidity at two time intervals during incubation (Figure 1A). Over time, solidity increased on all matrices with Geltrex and Matrigel changing at similar rates that were distinct from laminin-521. Colonies tend to spread more on laminin-521, which may account for their lower solidity. Examples of colonies with very high and very low solidity are shown in Figure 1B-E. These data show that solidity is a useful feature for evaluating colony morphology, that solidity increased during 48 hours of culture, and that solidity varied on different substrates.

3.3 | Comparison of hESC attachment and growth on three substrates

To determine if attachment efficiency varied with different substrates, equal numbers of H9 hESC colonies were plated on Matrigel, Geltrex, or laminin-521, incubated 10 minutes, and washed to remove unattached colonies. Colonies were stained with DAPI, and the number of colonies on each plate was counted using a Nikon fluorescent microscope (Figure 1F). For our experimental conditions, significantly more colonies attached to laminin-521 than to Matrigel or Geltrex, and attachment was slightly but significantly more efficient on Matrigel than on Geltrex (Figure 1F).

The overall growth of colonies on the three substrates was evaluated by determining colony area (pixels²/well). After 72 hours of culture, area was significantly greater (P < .001) for the group on laminin-521 (Figure 1G). However, when time-lapse area data were normalized to 1 at the first time point, the fold increase in area over time was not significantly different between groups (Figure 1H), suggesting that better attachment to laminin-521 (Figure 1F), not faster growth rate, accounted for the increase in area seen in Figure 1G.

3.4 | Evaluation of cell death on different matrices using StemCellQC

Dead hESCs are extruded from colonies and usually remain attached to the colonies’ surface throughout incubation (Figure 2A-D yellow arrows). In phase contrast micrographs, the extruded dead cells appear bright, and they are easily distinguished by their intensity from healthy cells within the colony. This phenomenon is shown in Figure 2E-P at four times during culture for colonies grown on the three substrates. The cells on Matrigel and Geltrex had more bright dead cells on their surfaces (Figure 2E-L) than those on laminin-521 (Figure 2M-P). Death of the surface cells was confirmed using MitoSOX Red, a fluorescent indicator that reports on superoxide levels, and trypan blue, a stain that enters dead cells. The cells on the surfaces of colonies were positive for both MitoSOX Red and trypan blue, while viable cells within the colony were unlabeled (Figure 2Q,R).

The brightness/area ratio feature in StemCellQC can be used to quantify cell death in PSC colonies. StemCellQC analysis of time-lapse data confirmed that colonies grown on both Geltrex and Matrigel experienced significantly more cell death during culture than those on laminin-521 (Figure 2S). The brightness/area ratio was relatively constant during incubation on laminin-521 (Figure 2S). The brightness/area ratio for Geltrex was elevated throughout incubation relative to the other two matrices but did begin decreasing at about 32 hours. The brightness/area ratio on Matrigel was significantly higher than on laminin-521 throughout incubation, although it decreased over time (Figure 2S). The brightness/area ratio data demonstrate that more cell death occurred during culture on Matrigel and Geltrex than on laminin-521, which could also contribute to the higher yield of cells seen on laminin-521 (Figure 1G). While most dead cells appear to remain attached to the colonies and there are clearly significant differences between colonies on different matrices, we cannot exclude the possibility that some dead cells detached from colonies leading to an underestimation of cell death.

3.5 | Effect of culture media on morphology, growth, death, and motility

To determine how colony behavior was affected by different culture media, H9 cells were plated on Matrigel and grown in mTeSR1-A or mTeSR1-B (batches purchased at different times), E8, and mTeSR2.

Solidity was similar in all media except for mTeSR1-B, which produced colonies with significantly lower solidity than the other media during the first 16 hours of incubation (Figure 3A). These colonies...
FIGURE 4  Legend on next page.
exhibited a general decline in roundedness (solidity) from hours 0 to 11, after which they recovered and by the end of the incubation were similar to the other groups. The solidity of colonies in the other three media increased at a similar rate over the entire culture period and were not significantly different from each other.

Growth was determined by quantifying normalized colony area (fold increase) and number of protrusions, which correlate with growth on Matrigel. Significance was determined by comparing each medium to mTeSR1-A. Area and protrusions were similar, but not identical, for the H9 cells grown in different media (Figure 3B,C). Compared with mTeSR1-A, cells grew significantly faster in mTeSR2 and E8 and significantly slower in mTeSR1-B. Except for the mTeSR2 protrusion group, the standard errors of the mean were small and remarkably similar in all groups, indicating very uniform growth among colonies in each group.

In all groups, the brightness/area ratio decreased with time in culture indicating that colony growth outpaced cell death (Figure 3D). The H9 colonies grown in mTeSR1-B had the highest brightness/area ratio indicating that this group experienced more cell death than groups in the other three media. Cell death was significantly greater in both E8 and mTeSR1-B, than in mTeSR1-A.

While colony motility was similar in all culture media, the total distance traveled was significantly less in E8, mTeSR2 and mTeSR1-B than in mTeSR1-A. Total displacement was also significantly lower in mTeSR1-B than in mTeSR1-A. The SEMs were small for both motility features, indicating all colonies in each group behaved similarly (Figure 3E,F).

Taken together, these data show that significant differences were observed among different media for all features analyzed, indicating that media choice can affect colony behavior. Furthermore, the results with mTeSR1-A and mTeSR1-B show that different batches of the same product can vary.

### 3.6 Comparison of different PSC lines

StemCellQC was used to compare the morphology and dynamic behavior of H9 hESCs, H1 hESCs, and CC3 iPSCs on Matrigel in mTeSR1-A. The H9 line maintained a consistently high level of solidity throughout 70 hours of culture, indicative of normal circular morphology (Figure 4A). Solidity for the H1 and CC3 colonies was similar through about 40 hours of culture after which CC3 solidity increased, while H1 leveled off and diverged from CC3. The CC3 colonies demonstrated the largest gain in solidity of the three lines. The initial solidity of both H1 and CC3 cells was significantly lower than that of the H9 hESCs.

When comparing area, H1 and CC3 were similar, while the H9 line grew significantly faster (Figure 4B). The variance within each group was remarkably small.

The initial and final brightness of H9, H1, and CC3 colonies are shown in the enhanced micrographs with the H9 colonies displaying less brightness than the H1 and CC3 at both timepoints (Figure 4C-H). StemCellQC confirmed quantitatively that the H9 line had a lower brightness/area ratio than the other two lines throughout 70 hours of culture (Figure 4I). H1 colonies initially had low brightness/area ratios, which increased over time, and by the end of incubation were about double that seen in the H9 colonies. The CC3 colonies had initially high brightness/area ratios, which decreased until about 35 hours, after which the ratio increased until the end of incubation. The variance in the H9 group was considerably less than in the other two groups.

Total distance traveled and total displacement were evaluated for the three cell lines grown on Matrigel. Examples of traveled paths are shown for each cell type in CL-Quant generated images (red lines, Figure 4J-L). StemCellQC analyses showed that the total distance traveled was significantly different for the three cell lines (Figure 4M). H9 were the least motile, CC3 were intermediate, and H1 traveled the greatest total distance. A similar relationship was observed for total displacement with the H1 cells having significantly larger total displacement than the H9 cells (Figure 4N). The SEMs were small indicating that motility was uniform for all colonies within each group.

The H9 line differed from the other two lines in a manner that is consistent with better in vitro vigor. The H9 colonies maintained consistently lower brightness/area ratios, were more circular (higher solidity), and grew faster than H1 and CC3 colonies. The lower motility in the H9 group is characteristic of larger colonies.11

### 3.7 Assessment of two H9 hESC phenotypes

The phenotypes of H9 hESCs can change when cultured in different labs. The colony in Figure 5A,B has small tightly packed cells, which
**FIGURE 5** Legend on next page.
we refer to as the “small phenotype,” and is characteristic of colonies freshly plated in our lab from a frozen WiCell vial (Figure 5A,B). Upon culture in a different lab, these colonies acquired a “large phenotype” characterized by larger cells with spaces between cells (Figure 5C,D). The large phenotype is very robust and can grow in conditions that do not support the small phenotype. The small phenotype can form neural rosettes that differentiate into neurons (Figure 5E,F), while the large phenotype fails to form neural rosettes and neurons in differentiating culture medium (Figure 5G,H). The dynamic characteristics of the two phenotypes was compared using StemCellQC.

Colony solidity for the large phenotype began and ended at a higher ratio than for the small phenotype with the solidity increasing during incubation at similar rates for both phenotypes (Figure 5I). Values were not significantly different at any time, although they often came close to significance.

The area and protrusion features were similar for both phenotypes with the small phenotype growing slightly faster at the end of incubation (Figure 5J-K). For both area and protrusions, the standard errors of the means were small indicating all colonies within groups behaved similarly with respect to these two parameters.

StemCellQC identified differences in average intensity between the two H9 phenotypes (Figure 5L). Average intensity was similar within the two groups but differed significantly between groups. This difference occurred because large phenotype cells spread out more than small phenotype cells, and intensity decreased as spreading increased.

Colonies with the small phenotype were displaced a significantly greater total distance and traveled significantly further than the large phenotype (Figure 5M,N). Variation within the total displacement feature was remarkably small indicating very uniform displacement of all colonies within each group.

3.8 Dynamic differences in undifferentiated and differentiating H9 hESC colonies

Micrographs of H9 hESCs labeled with DAPI and OCT4 illustrate that these colonies were undifferentiated in culture (Figure 6A,B). Healthy H9 undifferentiated colonies had a round shape with small tightly packed cells that stain strongly for nuclear OCT4 and DAPI (Figure 6C). Differentiating H9 colonies had larger individual cells separated by bright spaces and an accumulation of bright dying cells in the middle of the colony (Figure 6D). The nuclei in the differentiating colonies stained with DAPI, but most lacked nuclear OCT4 labeling (Figure 6D).

StemCellQC detected dynamic differences in the undifferentiated and differentiating colonies. There was a significantly greater increase in colony area in the undifferentiated group vs the differentiating group (Figure 6E). The increase in area in the differentiating colonies indicates that some cell division was occurring in this group, but the rate of growth was less than in the undifferentiated colonies.

Brightness features were evaluated for the two groups of colonies. Overall average intensity (0-255) was significantly higher for the differentiating colonies, which usually had numerous bright dying cells. A similar collection of bright dead cells was not observed in undifferentiated colonies (Figure 6A vs C). The average intensity increased slightly in both groups during incubation (Figure 6F). The variance in the differentiating group was much larger than in the undifferentiated group, indicating that the accumulation of dead cells varied on the differentiating colonies. Similar results were obtained for the brightness/area ratio, which increased over time for the differentiating colonies (Figure 6G).

Total distance traveled for the undifferentiated colonies was significantly greater than for the differentiating colonies (Figure 6H). The differentiating cells migrating away from the periphery of the colony in Figure 6H may impede movement of the main body of the colony accounting for less total distance traveled by the differentiating colony.

3.9 The effect of an environmental chemical on three PSC lines

hESCs can be used to model epiblast cells and determine if environmental chemicals adversely affect early stages of development. Cinnamaldehyde is a flavor chemical used in consumer products, such as food and electronic cigarettes, which can have adverse effects on human cells. H9 hESCs, H1 hESCs, and CC3 iPSCs were treated with 7.6 × 10⁻⁶ mM cinnamaldehyde, a concentration that does not produce an effect in the MTT cytotoxicity assay, then analyzed using StemCellQC.

In the untreated controls, the perimeter for all three cell lines increased during 48 hours of incubation (Figure 7A,C,E). The control
**FIGURE 6** Legend on next page.
data showed that colony growth was greater in the H9 group than in the H1 and CC3 groups, which were similar to each other (Figure 7B). In contrast, perimeter increased at a slower rate in the three groups that received cinnamaldehyde treatment (Figure 7A), an effect that was strongest in the H1 group.

The brightness/area ratio in the three control groups changed only slightly during incubation (Figure 7B,D,F). In contrast, the ratio for all three treated groups increased significantly (Figure 7B,D,F), indicating an increase in cell death in the treated colonies. Other features that were evaluated were generally significantly different in the control and treated groups (Supplemental Figures 2-3).

4 | DISCUSSION

Label-free live-cell time-lapse imaging coupled with video bioinformatics analysis is a powerful technology that enables PSC colony morphology and dynamics to be quantified during in vitro culture. This approach provides both quality control and experimental data relevant to basic research, translational work, and toxicological testing. Our overall goal was to evaluate and demonstrate the usefulness of StemCellQC in discriminating differences in colony morphology and dynamic behavior in various experimental culture conditions and to compare the performance of various cell lines/phenotypes during in vitro culture. Our data show that analysis of time-lapse images with StemCellQC quantitatively distinguishes morphological and dynamic features in different pluripotent cell lines, experimental conditions, and batches of culture media. Differences in attachment to substrates and growth in culture media can be used to optimize culture conditions. Effects of environmental chemicals or drugs on hESCs, which appear to model epiblast cells, can be assessed quantitatively using StemCellQC. Solidity, area, protrusions, average intensity, brightness/area ratio, total displacement, and total distance traveled were the most useful features. A remarkable finding of the study was the low within group variance in many of the experiments. For example, the area and motility features often had small SEMs, indicating that all colonies in a group performed similarly. Low variance has the important advantage of allowing statistical differences to be detected when comparing groups during data analysis.

Although growth was similar on all matrices, the yield of cells on laminin-521 was significantly greater than on Matrigel or Geltrex. Several factors account for this difference. Almost twice as many hPSC colonies/cells attached to laminin-521. This rapid attachment followed by increased motility of plated colonies enabled small colonies on laminin-521 to locate and merge with each other, a factor that promotes colony survival and improves plating efficiency. The lower death rate on laminin-521 also contributed to the higher yields on this substrate. Laminin-521 has the further advantages of being available in recombinant form enabling xeno-free culture and permitting clonal expansion of hESCs in conjunction with e-cadherin. These advantages of laminin-521 could be significant in laboratories where time and resources are a factor and optimizing yield is important. While not tested in this study, other matrices that are also used in PSC culture, such as recombinant vitronectin, could be evaluated using StemCellQC.

StemCellQC can be used to compare batches of culture media. H9 hESCs grew similarly in three of the four media that were tested. The mTeSR1-B, which did not support pluripotency, produced results in solidity, brightness/area ratio, and total displacement that were different from the other three media (Figure 3G,H). Batches of mTeSR1 purchased after mTeSR1-B have shown improvement. Modern PSC culture has moved toward recombinant substrates and xeno-free media to avoid variation between batches. The use of bovine serum albumin (BSA) in mTeSR1 was a motivating factor in the development of Es medium, which lacks animal or human sourced proteins. In general, colonies in Es and mTeSR2 performed similarly to those in mTeSR1-A. StemCellQC can be used to monitor batch variation and ensure quality control in research and clinical laboratories where uniformity of culture medium is critical.

The three cell lines that were evaluated behaved differently in culture with the H9 line being distinct from the H1 and CC3 cells in several key features. H9 cells had a faster growth rate (area over time) and slower death rate. The morphological and dynamic characteristics of each cell line may need to be considered in experimental designs and monitored over time to determine if behavioral dynamics are altered with extended passaging. Other studies have documented differences in gene expression between ESCs and iPSCs and found variability in the differentiation potential of various hESC lines. Video bioinformatics analysis of the morphology and dynamic behavior in culture adds another dimension to these technologies that can be used to compare cell lines and monitor lines over time. Once the normal characteristics of a cell line are established, subsequent monitoring would reveal if a change in behavior occurs with repeated passaging or experimental treatment. Each lab using StemCellQC would
FIGURE 7

Legend on next page.
need to establish baseline criteria for their PSC lines in order to make subsequent comparisons.

hESC colonies normally have defined borders and cells with a small cytoplasmic to nuclear ratio. Although labs culturing hESCs are often aware that the phenotype of hESCs can change during culture, the significance of these changes is not well understood. Our comparison of large and small phenotype hESC colonies showed that growth rate was similar but solidity, average intensity, and motility were different in the two groups. The greater spreading of the large phenotype may contribute to their lower average intensity and reduced motility, which decreased as colonies grew. The failure of the large phenotype to differentiate into neurons suggests that they are not truly pluripotent.

The use of hESCs in drug and toxicological testing is an important underused application, and new methods are continually being evaluated. Treatment of hESCs and iPSCs with cinnamaldehyde showed that toxicity can be quantified using StemCellQC and five of the six features quantified with StemCellQC were significantly affected by a very low concentration of cinnamaldehyde. The three cell lines showed similar responses to treatment with the CC3 iPSCs and H1 hESCs being somewhat more sensitive than the H9 hESCs. The low variances within each cell line for most features make them valuable for toxicological and drug testing.

Live-cell imaging of hESCs is an emerging field with numerous potential applications, and several approaches have been developed to evaluate time-lapse data derived from PSC. Others have used live-cell imaging to characterize bottlenecks to colony formation when single hESCs are plated at low density. A “big data” approach used live-cell imaging to distinguish OCT4 expressing colonies. Label-free live-cell imaging of reprogrammed colonies has been used to identify small colonies that will go on to form iPSCs. Time-lapse imaging has also been used to study cells that are undergoing reprogramming to better understand the dynamics of the reprogramming process. Because of their small cytoplasmic to nuclear ratio, the number of cells in hESC colonies is difficult to determine, but cell counting in colonies has been successfully done using label-free time-lapse images. New PSC culture media are continually being developed and tested with hPSC, as well as with embryonic stem cells from other species, and StemCellQC could be used to help evaluate the performance of such media. StemCellQC provides a novel video bioinformatics tool that will complement existing methods for the analysis of complex time-lapse PSC data. Application of StemCellQC to PSC projects can improve culture, help maintain quality control, and expand experimental studies that can be done using PSC. While StemCellQC monitors colony morphology and dynamics, future software could focus on intracellular organelles and objects requiring higher resolution. Toward this end, we recently introduced MitoMo software that analyzes mitochondrial morphology and dynamics in single cells. While similar analysis will be challenging in small hESCs, future development and extension of StemCellQC could include organelle analysis.

5 | CONCLUSION

StemCellQC provides a rapid, resource-saving, noninvasive method for quantitatively analyzing the morphology, quality and behavior of cultured hPSCs for basic research and therapeutic use. Colony solidity, growth, cell death, protrusions, intensity, and colony motility were useful features that varied with different experimental conditions, culture media, cell lines, colony phenotype, and toxicant treatment. Analysis can be done using label-free phase contrast images and does not require the use of fluorescent or colorimetric probes that may affect data. StemCellQC, while used in this study with PSCs, could be applied to any type of cell that grows in colonies.

StemCellQC has many potential applications in stem cell laboratories. It could be used to: (a) assess new cell lines and identify those that are most robust and have desirable features, such as normal growth rate; (b) verify quality and show that cells have not changed during repeated passaging; (c) quantify and analyze video data; (d) compare new media and identify those that perform best; (e) monitor cell quality in translational and clinical labs, especially when preparing cells for transfer to patients; and (f) perform toxicological and drug testing.

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CONFLICT OF INTEREST
The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS
S.C.L.: conception and design, collection/assembly of data, data analysis and interpretation, manuscript writing; A.L.: collection/assembly of data, data analysis and interpretation, manuscript writing; L.A.: collection/assembly of data, data analysis and interpretation, manuscript writing and editing; P.T.: conception and design; data analysis and interpretation; manuscript writing; financial support; final approval of manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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