THRIVE BIOSCIENCE

Variability in Cell Confluency: Comparison of Human and CellAssist® Assessments

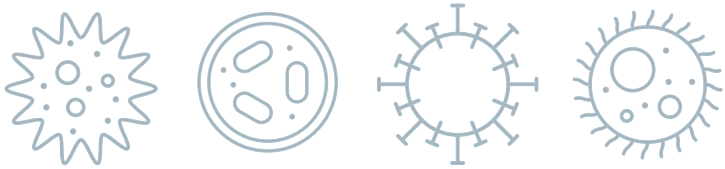
One of the greatest impediments to cost-effective drug development pipelines has been the frustrating realization that research reports of promising drug targets cannot reliably be reproduced in pharmaceutical laboratories [1-3].
How can two laboratories following the same procedure get such vastly different outcomes from cell-based studies? Possible causes contributing to this billion-dollar problem include human variability in experimental procedures, contaminated or incompletely characterized cell lines, poorly validated antibody reagents, inadequate study design, and even bias in reporting of data [3].

In this report, we examine human variability in cell confluency estimation, compare it to the objective confluency measurements performed by the Thrive Bioscience CellAssist, and describe potential impacts of human variability on cell-based assays. The data presented was gathered in a study employing a battery of test images evaluated by a population of cell biologists.

Background

A source of variability in cell-based studies is the estimation of percent confluency, which is typically subjective and prone to individual variation. Confluency estimates are used as triggers for key events in cell culture including initiation of cell-based assays, transfection, and passaging. Cells entering the high confluency range (typically 70-95%) experience increasing cell-cell contacts which trigger gene expression, morphologic, and physiologic changes [4-6]. For example, NIH 3T3 cells undergo a distinct morphologic change, from elongated to cobblestone appearance, above 70% confluency [7]. Many procedures call for cell harvest or assay initiation at 80-85% confluency. However, some cell types require passaging at lower confluency (<80% and 50% for NIH 3T3 and Sol8 cells, respectively) to avoid inhibition of proliferation and differentiation [8-10].

Myoblasts and adipocytes undergo spontaneous differentiation as they approach confluency, and accurate confluency values are therefore needed to avoid the risk of including differentiated cells in frozen stocks [11]. In this context, confluency estimation accuracy within ±5% would seem to be acceptable for most research applications. In contrast, a confluency estimation inaccuracy or variability greater than ±10% between experiments or between users could logically be problematic, resulting in the research use of cells with significant physiologic differences.



Methods

Study Population

To better understand the variability inherent in manual estimation of percent confluency, a survey was conducted of 77 participants recruited at the 2017 ISSCR scientific congress. Participants were shown a panel of 11 de-identified images of commonly cultured cells at various densities and asked to estimate the percent confluency.

Cell culture and image analysis

For the purposes of this study, human HEK-293, HT-29 and HeLa cell lines were grown to various cell densities in culture-coated 6-well microplates using D-MEM media supplemented with 10% (v/v) fetal bovine serum. Images were captured with a digital imaging system developed at Thrive Bioscience employing a 4X Nikon objective. Percent confluency measurements were obtained using the CellAssist auto-confluency algorithm. In this report, percent confluency values are sometimes referred to as confluency units (c.u.) for convenience in data presentation. The 11 de-identified images presented to study participants included HEK- 293 cells (at 35 confluency units), HT-29 cells (at 21, 52 and 76 confluency units) and HeLa cells (at 24, 54, 84 and 88 confluency units). The 11 images in the study panel included three pairs of duplicates, at 35, 54 and 84 confluency units, presented to the viewer in orientations rotated 180°.

Auto-confluency reproducibility

To evaluate reproducibility of the CellAssist auto-confluency measurements, the three human cell lines were plated to generate cultures with medium or high confluency levels. Culture plate positioning and auto- confluency measurements were performed 5 independent times for the same field of view. The standard deviations (from 0.1 to 0.7 c.u.) indicated high overall reproducibility (Table 1).

Table 1
Reproducibility of CellAssist auto-confluency measurements (mean ± S.D.).

Medium Confluency				High Confluency			
HeLa	HEK-293	HT-29	HT-29	HeLa	HEK-293	HeLa	HEK-293
53.7 ± 0.2	55.8 ± 0.4	70.6 ± 0.3	72.8 ± 0.7	86.7 ± 0.2	95.3 ± 0.1	96.3 ± 0.1	97.1 ± 0.1

Results

Overall variability of human confluency estimates

Estimates of percent confluence by the study participants were analyzed for variability at each of the confluency levels, ranging from 21 to 88 confluency units, as determined by the CellAssist. Results are shown in Figure 1; the boxes represent the two quartiles of human confluency estimates closest to the median. The figure reveals high variability among human confluency estimates for each image, particularly for low confluency images below 70 c.u. For 6 of the 11 images, the two center quartiles of estimates had little or no overlap with the objective auto-confluency measurements. In general, manual estimates were more accurate and exhibited less variability at high confluency (84 and 88 c.u. images).

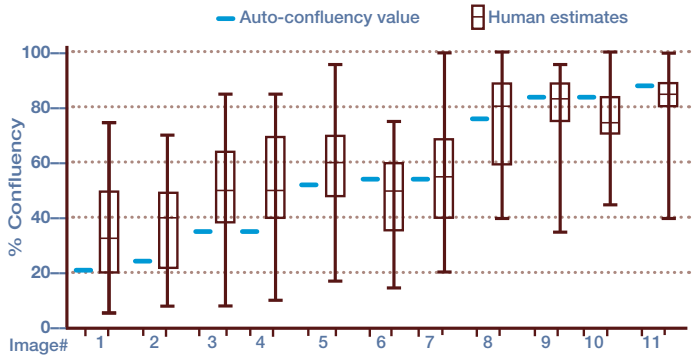


Figure 1 Variability (grouped by quartile) in human estimates of percent confluency relative to automated determinations

Consistency of participant estimates with duplicate images

In a laboratory setting, a researcher must be as reproducible as possible in order to obtain consistent and clearly interpretable results. The study design included a built-in test of personal reproducibility in confluency estimation. The 11 cell culture images included 3 pairs of identical images (at 35, 54 and 84 confluence units), presented to the viewer in orientations rotated by 180°. This exercise was intended to assess consistency of performance for each individual within a single work session. The differences between each individual's two estimates for each of the duplicate image pairs are shown as a histogram in Figure 2. In this figure, values are presented irrespective of the actual accuracy or inaccuracy of these estimates. Variability for individuals evaluating duplicate images was less than the variability observed across all images.

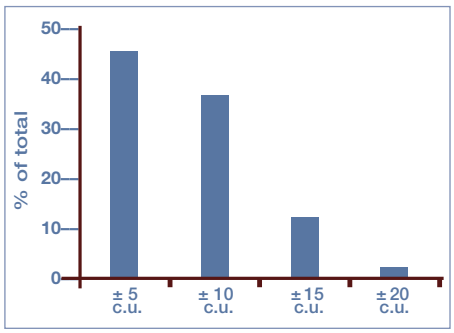


Figure 2 Overall, 46% of confluence estimates for duplicate images fell within a ±5 c.u. range and a further 37% fell within a ±10 c.u. range.

Variability around the mean of confluency estimates for pairs of identical images fell within a ±10 c.u. range.

How the Auto-confluency Algorithm can Overcome Human Variability

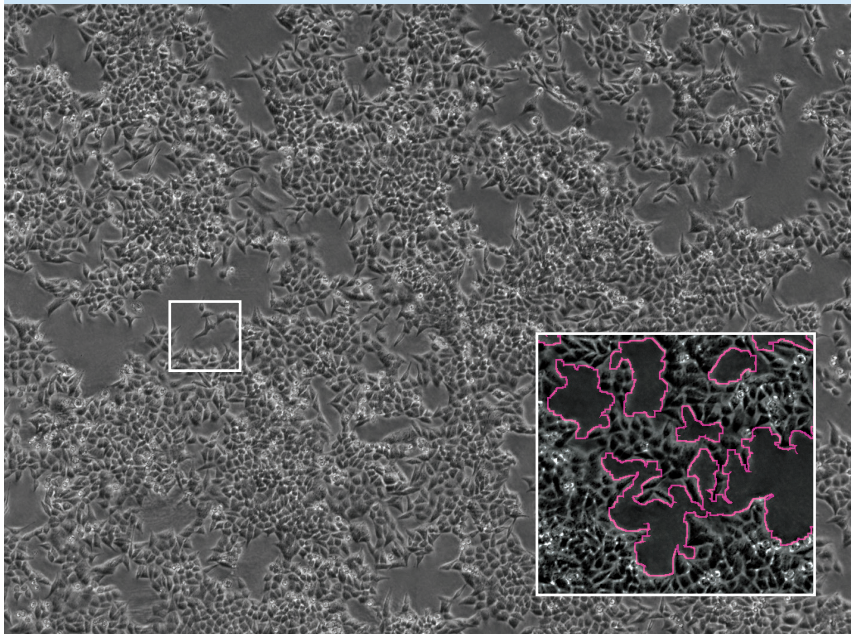
The CellAssist utilizes an auto-confluency module with proprietary auto-focus and image analysis algorithms to outline and quantify areas occupied by cells. Auto-confluency quantification is rule-based and highly consistent, whereas human subjective assessments can vary due to differences in training, experience, and pattern perception.

Two images from this study underscore this concern. Although the confluency values were similar (84% and 76%, respectively), study participants significantly and consistently over-estimated confluency of the 76% image. What could cause such a difference?

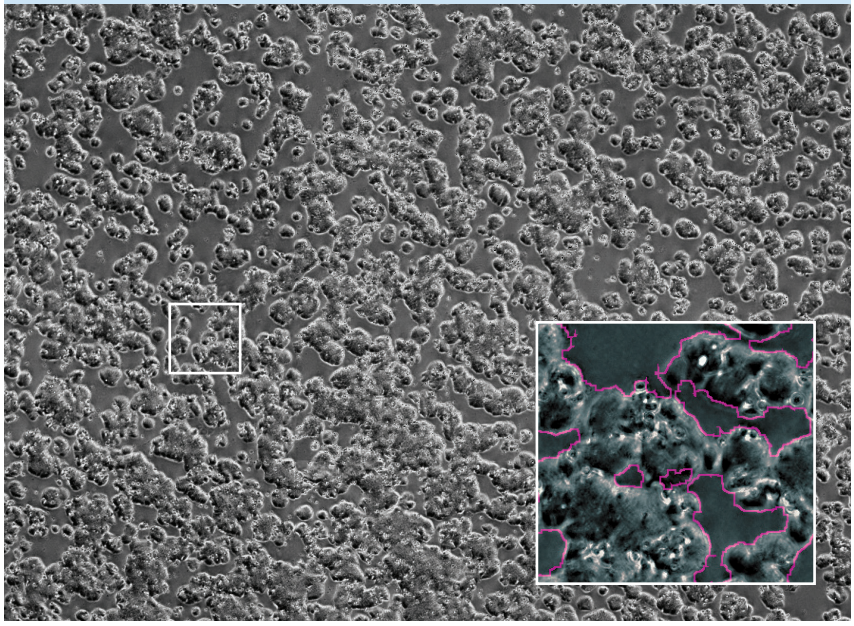
It is noted that the more uniform cell distribution of the 76% confluency image resulted in generally smaller areas between cell clusters. A high proportion of study participants scored the 76% image as 90% (Figure 1). Subjective assessments may be particularly prone to errors of this nature.

The CellAssist in certain imaging modes is able to evaluate confluence over the entire well. The CellAssist confluence measurements include cells seeded in areas that may be influenced by well edge effect and the CellAssist further reports the results by well region. This can provide more accurate representation than manual assessment typically sub-sampled in the center of the well.

84% confluency as measured by CellAssist
Median human estimate was 80%.
Only 36% of estimates were within ±5% of target.



76% confluency as measured by CellAssist
Median human estimate was 89%.
Only 24% of estimates were within ±5% of target.



Images: captured at 4X in phase contrast mode
Insets: show auto-confluence outlining at 4-fold further magnification

Discussion

Overall, results from this study suggest that high variability and inaccuracy persist in human estimates of confluency in cell culture. The most accurate and least variable estimates were generated at high confluency where most researchers may be well-practiced. Less variability was generally observed when paired duplicate images were evaluated by individuals. The results suggest that individual skill level varies widely and that subjective estimates are likely to fall far from actual confluency values. Furthermore, individuals skilled with one confluency range should not be assumed to be skilled in other situations. Thus, subjective, variable confluency estimates could plausibly contribute to widespread problems with reproducibility in cell-based research.

This preliminary study did not assess whether negative biological consequences result from variability and inaccuracy of confluency estimates. However, in our experience with immortalized cell lines, a confluency estimation error of 10-15 confluency units at low or medium confluence could easily result in a culture dish being 12 hours over- or under-grown at the intended time of harvest. For a cell-based assay protocol specifying growth to 80-85% confluency, errors of this magnitude could logically lead to variable results due to over-grown cultures with proportions of senescent cells.

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