Application Note

Cell Analysis

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Monitoring Cell Proliferation in Real Time Using the Agilent xCELLigence RTCA eSight

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Introduction

Cell proliferation is crucial to a myriad of biological processes. While proliferation drives organismal growth and the repair of injury, its dysregulation is associated with disease states such as autoimmunity[1] and cancer. In some areas of research, the proliferative capacity of cells is the direct object of inquiry. Examples of this include evaluating the impact that an exogenous cytokine or a gene knockout has on progression through the cell cycle. In contrast, cell proliferation is also commonly used as a generic indicator of cell health to shed light on some other phenomenon. Examples include tracking the proliferation of host cells as a way to detect the presence of a virus or using proliferation as a readout for drug toxicity. The difference between having proliferation be the primary subject of inquiry versus using it as a surrogate readout is not trivial. In the latter case, proliferation is often used by convention, even though it only functions as a blunt probe when more nuanced information about cell health would be advantageous.

Across the broad spectrum of contexts in which proliferation assays are employed, they have historically been, and continue to be, monitored predominantly using endpoint assays. The major drawbacks of these legacy approaches are 1) a significant amount of hands-on time is required, 2) only a limited number of data points are provided, and 3) tracking the number of cells that are present provides only a limited perspective on cell health.

Requiring only a cell seeding step, the Agilent xCELLigence RTCA eSight instrument monitors proliferation continuously via live-cell imaging. Working in a label-free format, eSight can track proliferation using percent confluence. Alternatively, when using red, green, or blue fluorescent labels, eSight counts the actual number of cells present. Recognizing that proliferation is often used as a general means of assessing cell health and acknowledging that changes in percent confluence or cell number are just one facet of cell health, eSight couples image acquisition capabilities with the simultaneous monitoring of cellular impedance. Interrogating

the same population of cells that images are being acquired from, impedance biosensors provide a composite readout of cell number, cell morphology, and cell-substrate attachment strength – yielding an orthogonal, more nuanced assessment of cell health and behavior. By combining the strengths of real-time impedance monitoring (simplicity, analytical sensitivity, and objectivity) with those of live cell imaging (specificity of the readout), eSight increases the information richness of the proliferation assay while simultaneously reducing the workload.

Assay principle

At the core of the eSight assay is a specialized electronic microtiter plate. Within the glass bottom of all 96 wells, a gold biosensor array continuously and non-invasively monitors cellular impedance. As shown at the bottom of Figure 1, the adhesion of cells to these biosensors impedes the flow of a microampere electric current – providing an exquisitely sensitive composite readout of cell number, cell size, and cell-substrate attachment strength. This cellular impedance signal is recorded at a user-defined temporal frequency (every minute, every hour, etc.), and is reported using a unitless parameter called Cell Index. As cells proliferate, they cover more surface area on the biosensor array, and the Cell Index rises. While impedance is being monitored, a viewing window in the center of the same well enables eSight to track cell proliferation via live-cell imaging in brightfield and three (red, green, blue) fluorescence channels (Figure 1).

Although cell proliferation can be imaged on eSight using % brightfield confluence alone, using the Agilent panel of fluorescent labels makes it possible to track changes in actual cell numbers over time. eLive Red and eLive Green are nontoxic dyes, specifically formulated for continuous live-cell imaging. Present in the growth media throughout the entire course of the assay, these dyes diffuse through the plasma membrane and bind to dsDNA, which increases their fluorescent intensity >1,000-fold and causes nuclei to appear as distinct red or green foci. As an alternative, Agilent offers lentiviruses (eLenti Red, eLenti Green, and eLenti Blue) for producing stable cell lines that constitutively express nuclear-localized fluorescent proteins.

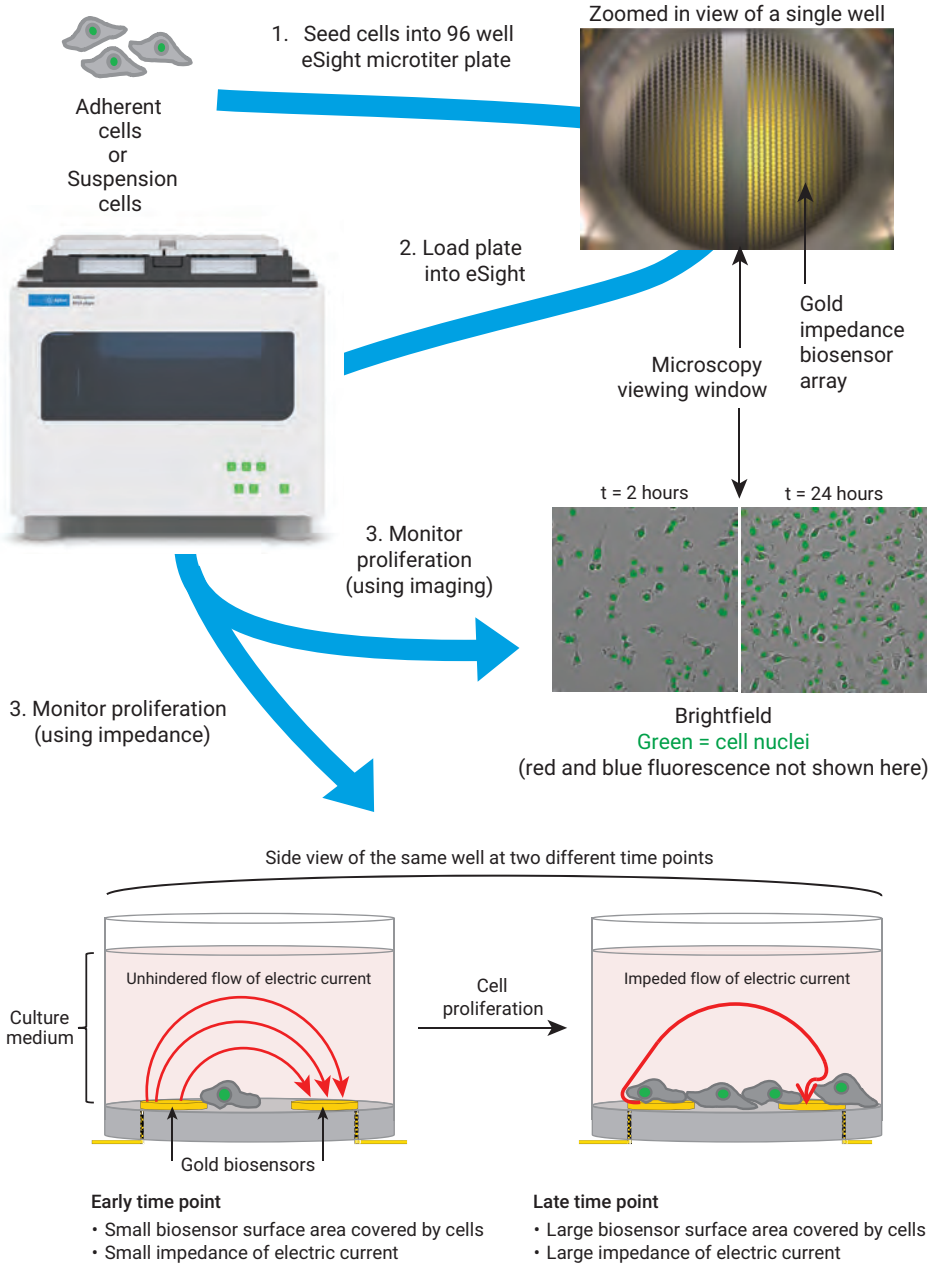

Figure 1. eSight workflow for cell proliferation assay.

Materials and methods

Cells, media, and growth conditions

HT-1080 cells (ATCC, part number CCL-121), HEK293 cells (ATCC, part number CRL-1573), and MDCK cells (ATCC, part number CCL-34) were cultured in EMEM (ATCC, part number 30-2003). MDA-MB-231 cells (ATCC, part number HTB-26) were cultured in DMEM (ATCC, part number 30-2002). A549 cells (ATCC, part number CCL-185) were cultured in F-12K media (Gibco, part number 21127-022). Co-culture of A549 and HT-1080 cells was done in a 1:1 mixture of EMEM/F-12k. For all the cell lines used in this study, growth media was supplemented with 10% FBS (Gibco, part number 16050-122) and 1% Pen/Strep (Hyclone, part number SV30010). Cell maintenance and assays were conducted at 37 °C/5% CO_2.

Fluorescently labeling cells

Although they can be added to the cells at any point before or during an assay, herein Agilent eLive Red (part number 8711040) and eLive Green (part number 8711039) dyes were added directly to E-Plate wells 18 hours post cell seeding to achieve a final concentration of 1×.

For the production of stable fluorescent cell lines, host cells were transduced with Agilent eLenti Red (part number 8711011), eLenti Green (part number 8711010), or eLenti Blue (part number 8711012) at a multiplicity of infection of three. To select for positive transductants, cells were grown in the presence of 2 µg/mL puromycin from day 3 to day 17 post-transduction.

Cell proliferation assays

All assays were conducted in Agilent E-Plate VIEW 96 microtiter plates (part number 300601020). After adding 50 µL of growth media to each well, a background impedance reading was made using eSight. 100 µL of cells were then added to each well to achieve the indicated seeding density. After letting the cells settle at room temperature for 30 minutes the E-Plate was loaded back into the eSight, impedance was then recorded every 15 minutes and images acquired every two hours. Four fields of view were captured for each channel being used. While brightfield exposure time was automatically optimized by the eSight software, exposure durations for the red, green, and blue channels were ~300 ms, ~300 ms, and 100 ms, respectively.

Results and discussion

Label-free cell proliferation monitoring

To demonstrate the ability of the eSight to track proliferation in a label-free manner, HT-1080 fibroblasts were seeded at a density of 3,000 cells/well. Images were then acquired every 2 hours and impedance was measured every 15 minutes. When assayed under "standard" conditions including 10% FBS, the cells displayed a distinct phase of adhesion and spreading over the first ~7 hours, followed by clear proliferation extending out to 48 hours (Figure 2A). As expected, the proliferative capacity of the cells correlated with the concentration of serum present in the growth medium (Figure 2B, upper panels). To quantify this serum-dependency, the eSight software was used to calculate the percent brightfield confluence as a function of time. While eSight's confluence masking is shown in the lower panels of Figure 2B, the proliferation time courses are plotted in Figure 2C, and the eSight-calculated doubling times are shown in Figure 2D. Monitoring the same population of cells that the images were acquired from, the impedance biosensors in the E-Plate wells also effectively captured the serum-dependency of HT-1080 proliferation (Figure 2E). Although this trend in serum dependency is similar when using impedance and imaging, the specific doubling times calculated from these two outputs differ slightly (Figures 2D and 2F).

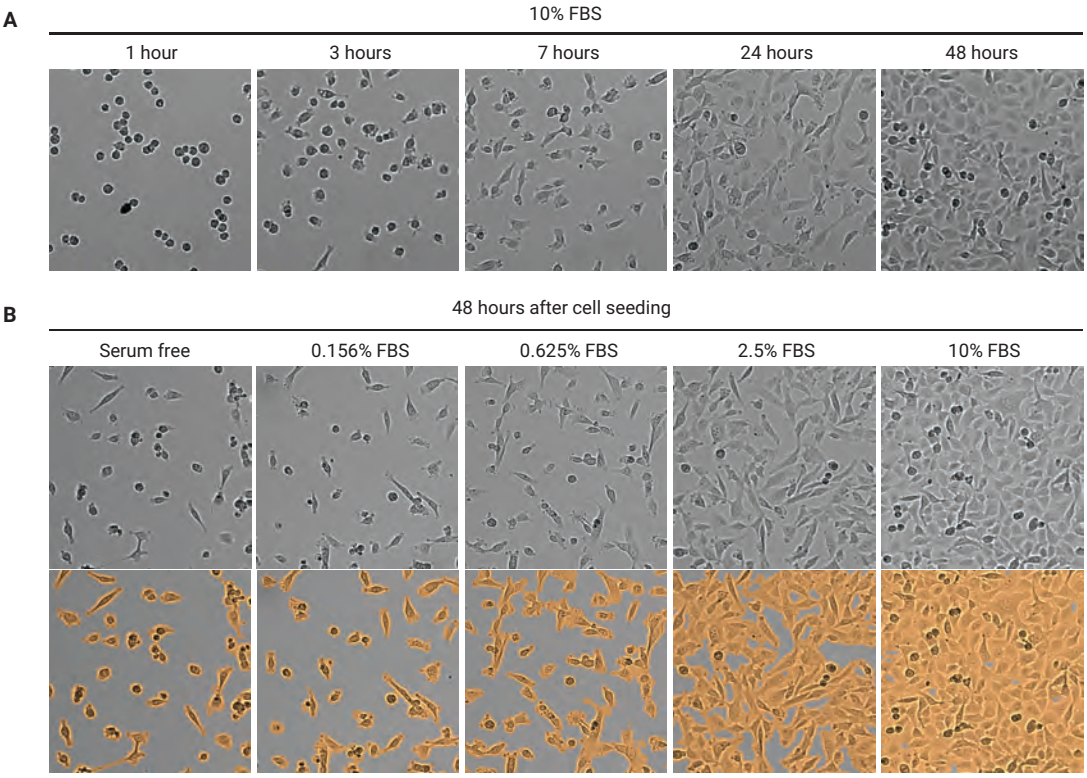

Figure 2A,B. Label-free monitoring of HT-1080 cell proliferation using an Agilent xCELLigence RTCA eSight analyzer. (A) Images tracking the progression of cell adhesion/spreading and proliferation over time. (B) Images from the 48 hour timepoint, highlighting the serum dependence of proliferation. Lower panels are the same as the upper panels, but with eSight confluence masking displayed in orange.

If imaging and impedance yield similar outputs, what is the value of using both to track proliferation? First, tracking proliferation simultaneously from two different perspectives provides a primary and confirmatory result all within the same assay, without increasing the workload. Second, impedance is a composite readout that reflects changes in cell number/morphology/attachment strength and therefore provides information that simply is not accessible by imaging alone. This is highlighted in Figure 3, which zooms in on the first 10 hours post HT-1080 cell seeding.

Whereas % confluence measurements are relatively insensitive to varying serum concentrations within this time frame (Figure 3A), impedance measurements capture, and perfectly rank order, the impact of varying serum concentrations (Figure 3B). That impedance is more sensitive than % confluence measurements in this early phase of the assay is attributable to the ability of the gold biosensors to detect changes in the strength with which cells are attached to the well bottom.

Collectively, the data in Figures 2 and 3 demonstrate that for a typical label-free proliferation assay, where changes in the number of cells present is the only concern, eSight's % confluence and impedance readouts both yield comparable data that is highly reproducible. However, in contexts where proliferation is merely being used as a generic surrogate for cell health and behavior, impedance provides a more complete and nuanced perspective. This is especially valuable in the context of manufacturing cell-based products, where the impedance signal can act as a critical quality attribute.

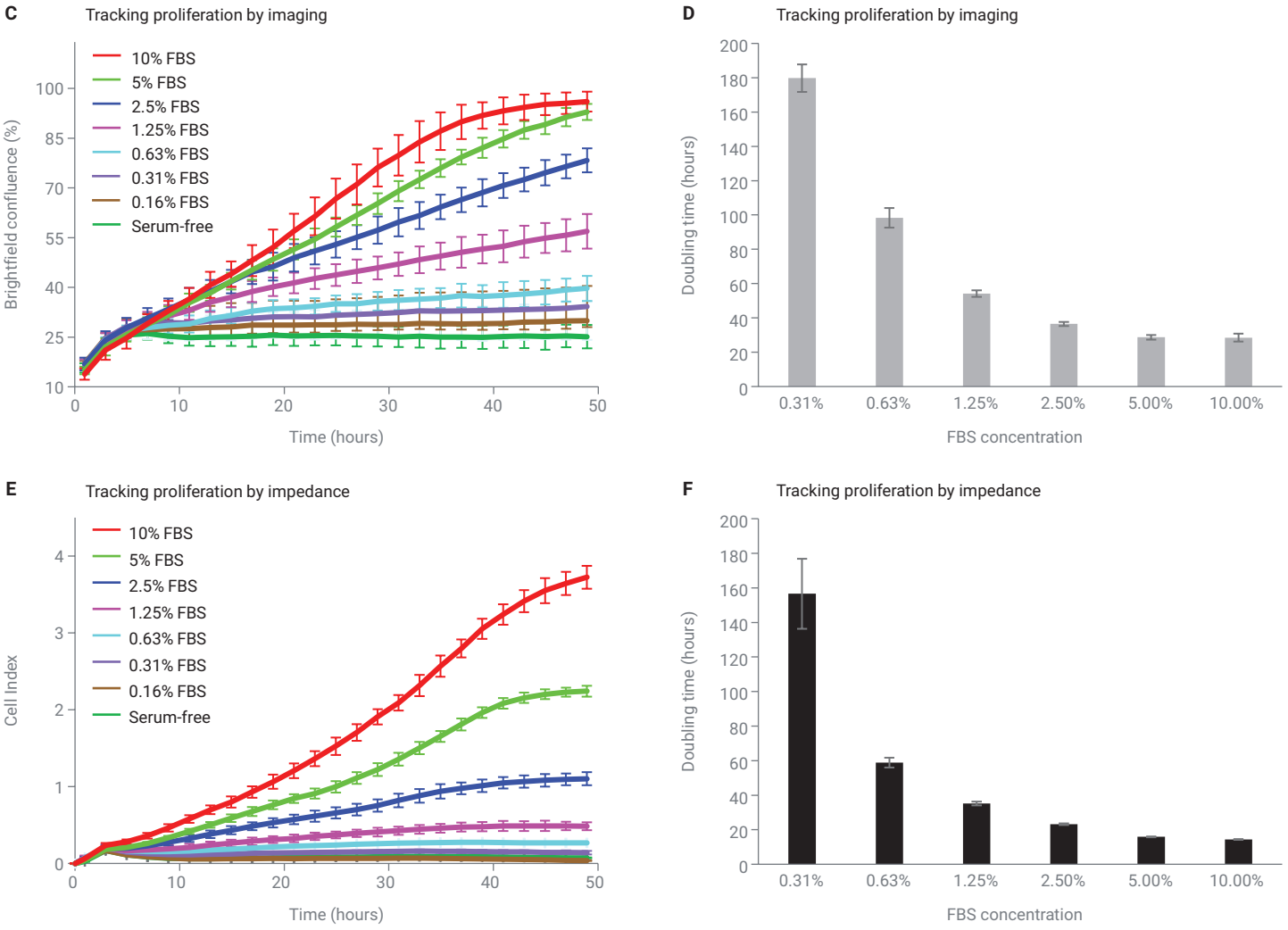

Figure 2C–F. Label-free monitoring of HT-1080 cell proliferation using an Agilent xCELLigence RTCA eSight analyzer. (C) Kinetics of HT-1080 proliferation as a function of serum concentration, using % brightfield confluence as the readout. (D) Working with the data traces shown in panel C, the eSight software was used to calculate the doubling time for the HT-1080 cells when grown at each serum concentration. (E) Kinetics of HT-1080 proliferation as a function of serum concentration, using impedance as the readout. (F) Working with the data traces shown in panel E, the eSight software was used to calculate the doubling time for the HT-1080 cells when grown at each serum concentration.

Using fluorescent dye-labeled nuclei to track proliferation via actual cell counts

As shown previously, in the context of a label-free assay eSight's image-based output is % confluence. As an alternative, when nuclei are fluorescently labeled eSight can provide actual cell counts. When included in the growth media, the Agilent eLive Red and eLive Green dyes display extremely low background fluorescence. In the presence of cells, these dyes diffuse through the plasma membrane and bind to dsDNA, which increases their fluorescence >1,000-fold (Figure 4A, upper panels) and enables distinct nuclei to be counted (lower panels of Figures 4A and 4B). Importantly, even when imaged at high temporal frequency over multiple days these fluorescent dyes are nonperturbing. As evidence of this, when analyzed by % brightfield confluence (Figure 4C) or impedance (Figure 4D), cells that are labeled with eLive Red or eLive Green display growth curves that are superimposable with those of unlabeled cells.

Consistent with what was observed in Figures 2D and 2F, the exact doubling time calculated by impedance differs slightly from that calculated using the image-based outputs: 9.4 hours (impedance), 14.6 hours (brightfield confluence), and 14.9 hours (fluorescent nuclei counts). These differences are rooted in the composite nature of the impedance signal which includes, but extends beyond, the number of cells present.

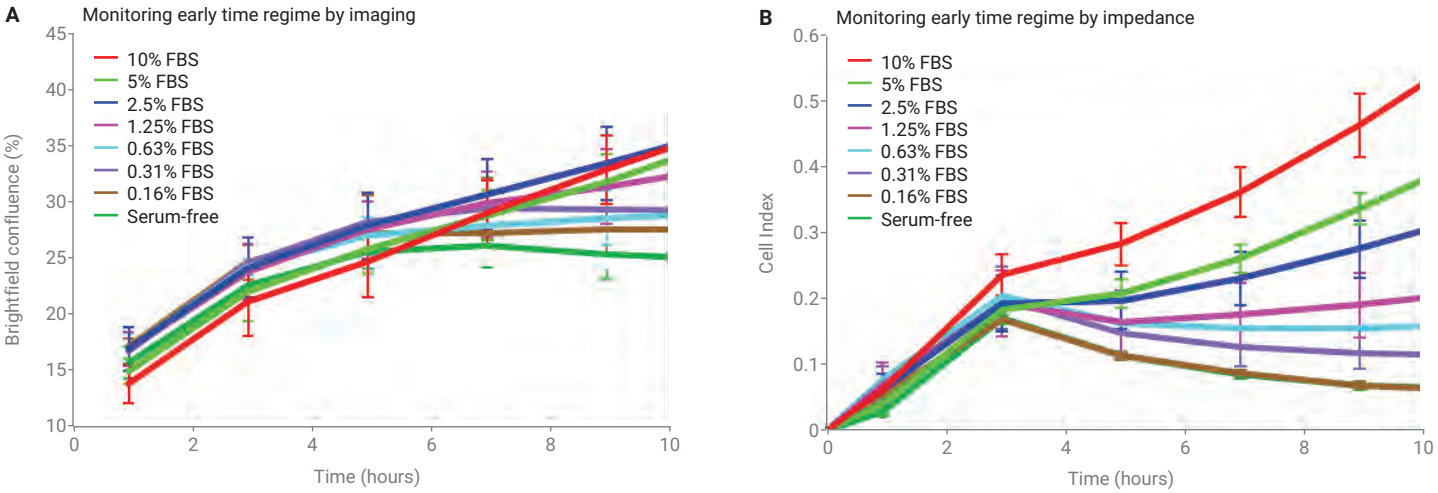

Figure 3. Evaluating serum dependency within the first 10 hours of a proliferation assay using imaging (A) and impedance (B). Error bars reflect the standard deviation for six replicate wells.

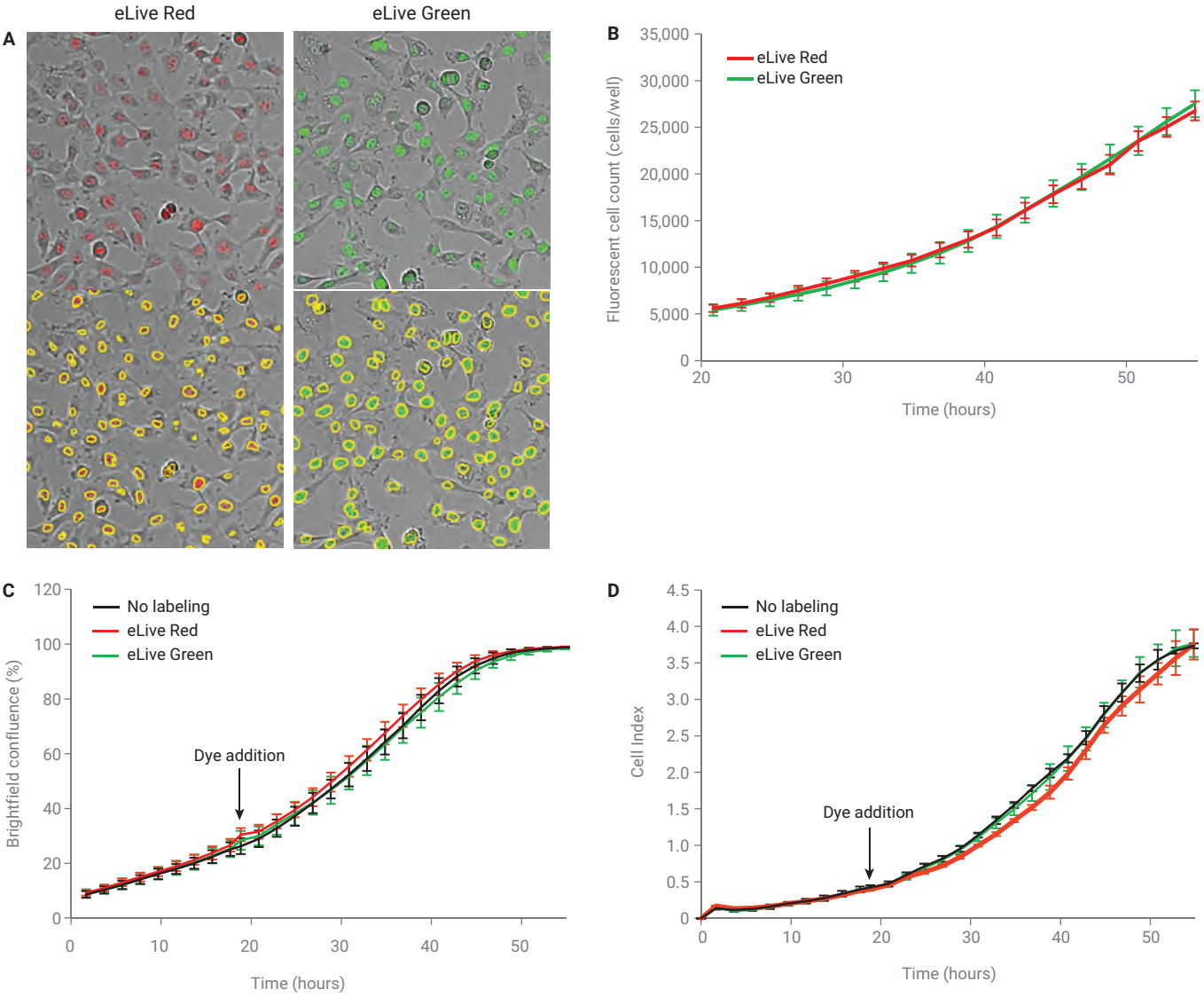

Figure 4. Tracking HT-1080 proliferation using nuclear-localizing fluorescent dyes to determine actual cell numbers. (A) Representative images of HT-1080 cells labeled with eLive Red and eLive Green. Lower panels are the same as those above, but include eSight masking to denote each fluorescent nucleus with a yellow outline. (B) Proliferation time courses using the number of fluorescent cells as the readout. Note that the first data point is 20 hours post cell seeding, because the eLive Red and eLIve Green dyes were not added to cells until 18 hours post cell seeding. (C) Comparison of proliferation kinetics, using % brightfield confluence as the output, for cells grown in the presence or absence of eLive dyes. (D) Comparison of proliferation kinetics, using impedance as the output, for cells grown in the presence or absence of eLive dyes. Error bars reflect the standard deviation for 12 replicate wells.

Using fluorescent protein-labeled nuclei to track proliferation via actual cell counts

As an alternative to transiently labeling cells with the eLive dyes, stable fluorescent cell lines can be produced using Agilent eLenti Red, eLenti Green, and eLenti Blue reagents. Upon integration into the host cell's genome, these lentiviruses employ an EF1α promoter to constitutively express nuclear-localized mKate, EGFP, or TagBFP, respectively. Example images, displayed with and without eSight fluorescent nuclei masking, are shown for three common cell lines in Figure 5A.

Using A549 cells that were previously transduced with eLenti Red, a simple comparison of proliferation kinetics as a function of cell seeding density (3k, 6k, or 9k cells/well) is shown in Figure 5B. Juxtaposing eSight impedance, % confluence, and fluorescent cell count outputs reveals a subtle yet important difference at the highest seeding density (9k cells/well). Whereas the impedance and % confluence curves begin to plateau at the 30 hour time point, the fluorescent cell count curve continues to steadily increase out through the 65 hour time point (Figure 5B). This difference is rooted in the fact that even after reaching 100% confluence the A549 cells do not immediately display contact inhibition and instead continue to proliferate, with cells progressively packing in more tightly next to one another (Figure 5C)*. This difference between eSight's outputs provides a more nuanced understanding than is possible with any single output alone and should be kept in mind as one output might be more useful/meaningful depending on the assay context and the question being addressed.

* The impedance signal plateaus when cells reach 100% confluence because the entire surface area of the gold biosensor array is now covered by a monolayer of cells. Although the A549 cells subsequently continue to proliferate, they pack more tightly laterally but do not increase the thickness of the monolayer, and thus the impedance signal remains constant.

Co-culture assays

It was demonstrated in a previous section that impedance, because it provides a composite readout of cell number/size/attachment strength, often captures subtleties of cell behavior that are inaccessible to image-based approaches. Conversely, because impedance interrogates the entire cell population as a whole, it cannot analyze the proliferation of distinct subsets of cells within a mixed population – which is something that image-based analyses excel at. As an example of this, A549 cells expressing EGFP were mixed

A Examples of different cell types stably expressing nuclear-localized red, green, or blue fluorescent protein

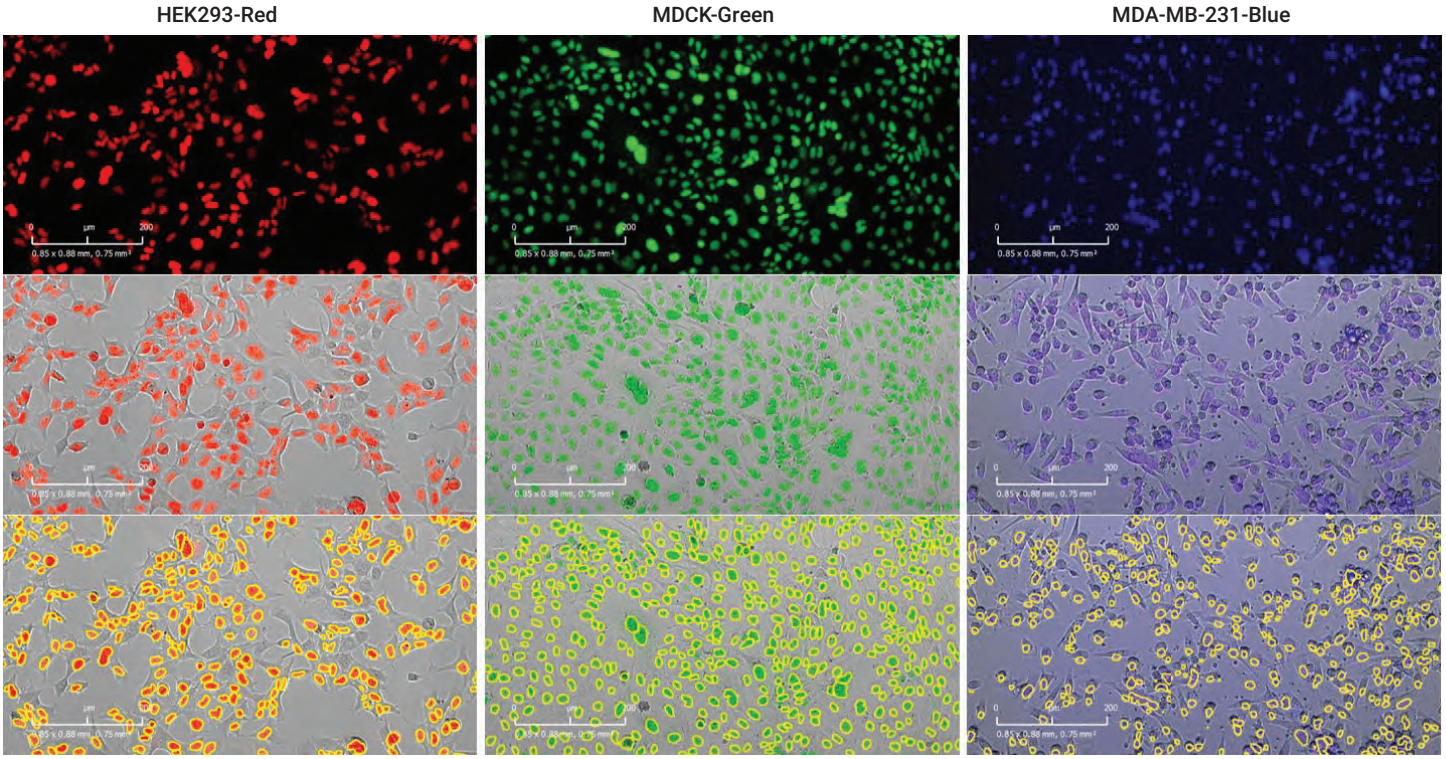

Figure 5A. Tracking cell proliferation using nuclear-localized fluorescent protein to determine actual cell numbers. Example images for HEK-293, MDCK, and MDA-MB-231 cells transduced with Agilent eLenti Red, eLenti Green, and eLenti Blue, respectively.

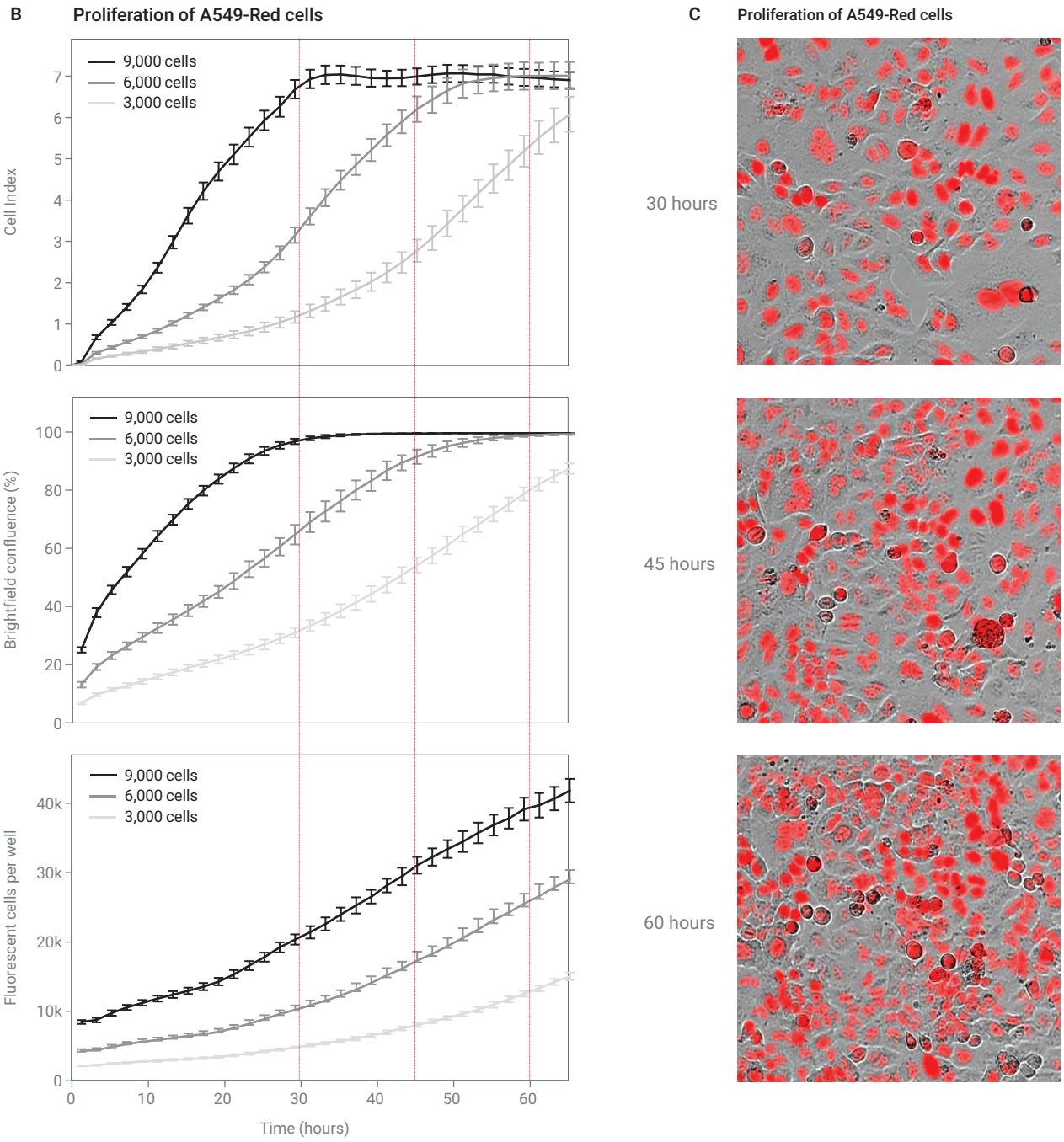

Figure 5B,C. Tracking cell proliferation using nuclear-localized fluorescent protein to determine actual cell numbers. (B) Proliferation kinetics, as a function of cell seeding density (3k, 6k, or 9k cells/well), for A549 cells that were previously transduced with eLenti Red. The vertical red dashed lines facilitate comparison of proliferation kinetics across eSight's three outputs of impedance, % confluence, and fluorescent cell counts. Error bars reflect the standard deviation for 12 replicate wells. (C) Although they are already confluent at the 30 hour time point, the A549-Red cells from panel B continue to proliferate, with cells packing more tightly next to one another as they progress through the 45 and 60 hour time points.

with HT-1080 cells expressing mKate at a 1:1 ratio before seeding in E-Plate wells. Through the simple application of red and green nuclear recognition masks, eSight clearly demonstrates the proliferative advantage that the HT-1080 cells have over the A549 cells within this mixed population (Figures 6A and 6B). This same approach can readily be applied to track the proliferative properties of each cell type present in studies of the tumor microenvironment, immune cell-mediated killing, etc.

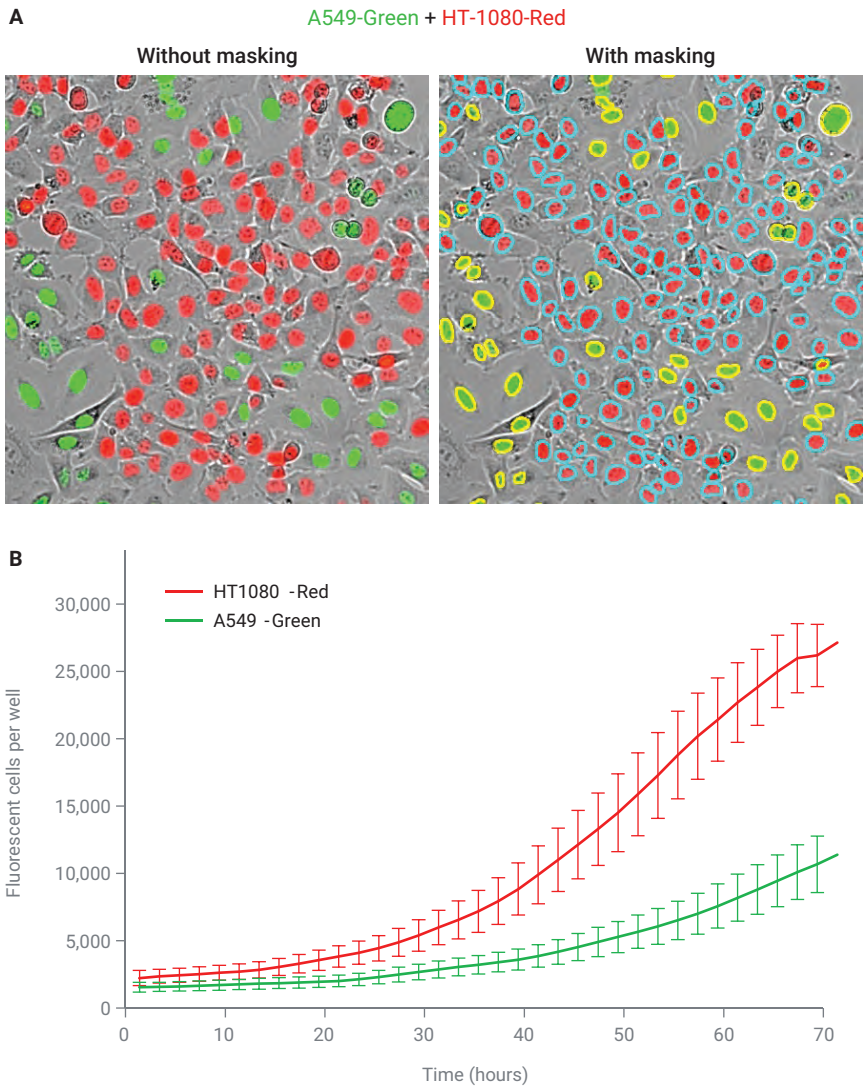

Figure 6. Image-based monitoring of proliferation for distinct cell types within a mixed population of cells. (A) Left panel: Image of EGFP-expressing A549 cells mixed with mKate-expressing HT-1080 cells. Right panel: Same as left panel but with eSight fluorescent nuclei masking applied (yellow outline around green nuclei and aqua outline around red nuclei). (B) Proliferation of A549 and HT-1080 cells within this co-culture context. Error bars reflect the standard deviation for 96 replicate wells.

Evaluating the accuracy of eSight's fluorescent cell counts

Data shown in the previous sections clearly demonstrates eSights's ability to track changes in the number of cells over time. To evaluate the accuracy of these fluorescent cell counts, eSight assays were run in parallel with manual cell counts on a hemocytometer and automated cell counts on a Luna-FL instrument. 5,000 A549 cells, which had previously been transduced with eLenti Blue, were seeded in E-Plate wells. At the 24, 48, 72, and 96 hour time points eSight images were collected and then the E-Plate was removed from the instrument and cells were harvested from select wells by trypsinization before manual or automated cell counting. The strong correlation between eSight-derived cell counts and those of the hemocytometer and automated cell counter provide confidence in the accuracy of eSight's image-based output. Note, also, the high reproducibility of the eSight counts, where the coefficient of variation for the 24, 48, 72, and 96 hour time points was 12.5%, 6.3%, 4.7%, and 5.9%, respectively.

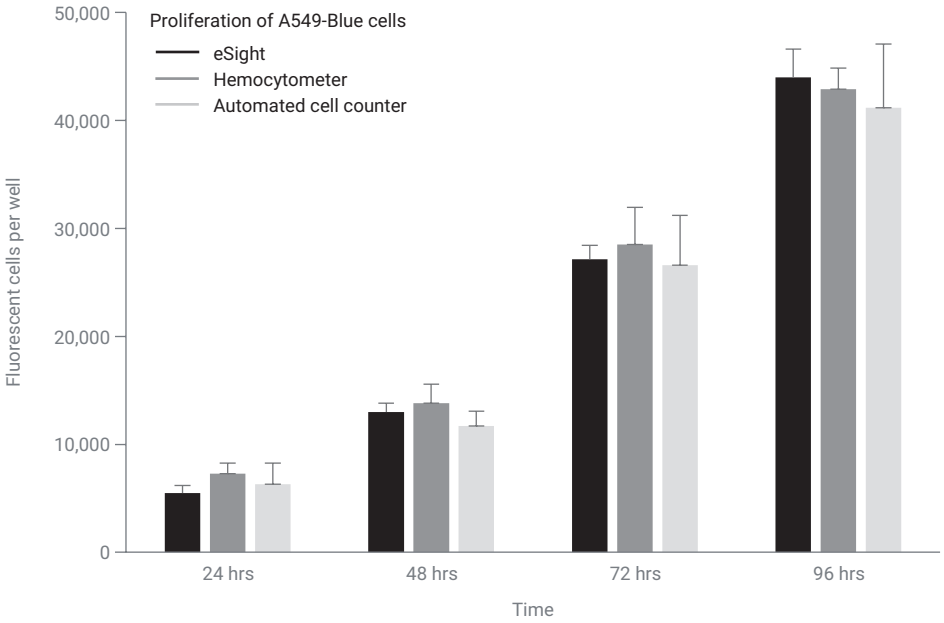

Figure 7. Comparison of cell counts from eSight, a hemocytometer, and an automated cell counter. Error bars reflect the standard deviation for duplicate wells.

Conclusion

The distinguishing features of the eSight proliferation assay described here are its simplicity and information richness. Following a simple cell seeding step, eSight tracks proliferation continuously and requires zero additional hands-on time. This stands in stark contrast to traditional methods where cells must be collected and processed at each time point of interest. eSight makes comparative analyses of proliferation both easier and more accurate because the continuum of data points enable one to select the most appropriate time point or time ranges to focus on. For example, consider the % confluence plot in Figure 5B. The real-time traces make it clear that comparisons of proliferation rates between the different seeding densities should be conducted before the 30 hour time point, after which the cells seeded at 9k/well are confluent. This type of distinction can go unnoticed in endpoint assays where one only has a few data points to work with.

Confirmation of a result using an orthogonal approach is the gold standard across science but is often skipped due to the time and/or cost involved. The impedance and imaging outputs of the eSight provide both a primary result and a confirmation of that result all in one assay, with both analyses derived from the exact same population of cells. This additional information is gained without any increase in the hands-on time required. The vast majority of the time, the impedance-based and image-based readouts correlate very well with one another. In instances where they do not, such as in Figure 5B where cell numbers continue to increase even after impedance plateaus due to the cells reaching confluence, this leads to a more rich and nuanced understanding of the cell's proliferative behavior.

Finally, the composite nature of the impedance readout makes it extremely sensitive to subtle cell behaviors that go undetected during typical proliferation assays. The ability to expose these behaviors, such as altered cell-substrate adhesion strength (Figure 3), makes the impedance readout especially useful in contexts where one is attempting to observe a cell's phenotypic response to a treatment and would like for this to include, but also extend beyond, mere proliferative behavior. These types of impedance-based information can be used, for example, as critical quality attributes in the context of manufacturing cell-based products.

Reference

1. Jones, J. L. *et al.* Human Autoimmunity after Lymphocyte Depletion is Caused by Homeostatic T-Cell Proliferation. *Proc. Natl. Acad. Sci. USA* **2013**, *110(50)*, 20200–5.

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