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Technical Note

EVE Analytics

OBJECTIVE

Nanolive's label-free technology makes it possible to image cells for long periods of time, at high temporal resolution. The quantity and complexity of the images generated allows us to visualize biological processes in unprecedented detail, but also magnifies the challenges associated with image analysis. Manual image registration and analysis is impossible and so computer-aided processing must be used to harness data complexity[1]. In this technical note, we introduce the key elements involved in cell segmentation, which are essential to understand the novelty of EVE Analytics (EA), Nanolive's software solution for quantitative cell analysis. We then evaluate the performance of EA segmentation against fluorescence-based segmentation and compare how metrics produced by both approaches differ.

STATE-OF-THE-ART OF CELL SEGMENTATION

(1) Primary object detection using image thresholding

Cell segmentation relies on one simple but fundamental concept; cells must have a signal that is different from the background. To achieve this, most studies use contrast agents, which bind to specific molecular components of the cell, allowing cells to be differentiated from the background by simple thresholding. The most commonly used contrast agent is the nuclear stain DAPI[2], but this dye requires the sample to be fixed. Other nuclear dyes such as Hoechst[3] or DRAQ5[4], can be tolerated by living cells, but not for long, as their presence perturbs fundamental genetic processes[5], and they require excitation to generate fluorescence, which generates substantial phototoxic stress[6].

(2) Primary object detection to anchor (or seed) a secondary step of detection

Nuclear dyes make it possible to quantify the number of cells but do not provide a lot of information about the size/area/shape of the cell. Using cytosolic stains such as Cell Trace or cholera toxin B overcomes this limitation[7] but complicates analysis for two reasons. Firstly, the local quantity of the cell material they stain varies, and so the fluorescent signal intensity differs dramatically within a cell. Secondly, the variability in cell edge morphology makes it much more complex to separate touching cells, prohibiting a simple thresholding approach like the one applied to segment nuclear objects[8]. Instead, primary nuclei objects are used to anchor (or seed) a secondary step of detection[1]. When coupled with local thresholding, this double detection approach allows fine details to be segmented[9]. Successful analysis, however, does not diminish the risk of cyto- and phototoxicity posed by the use of fluorescent compounds.

EVE Analytics benefits from a new segmentation technique, developed specifically for Nanolive's label-free images. Primary object detection relies on signal preparation and advanced thresholding methods to detect the cellular object seed. This is then followed by a secondary step based on our refractive index (RI) signal, which allows to capture the fine boundaries of cellular objects.

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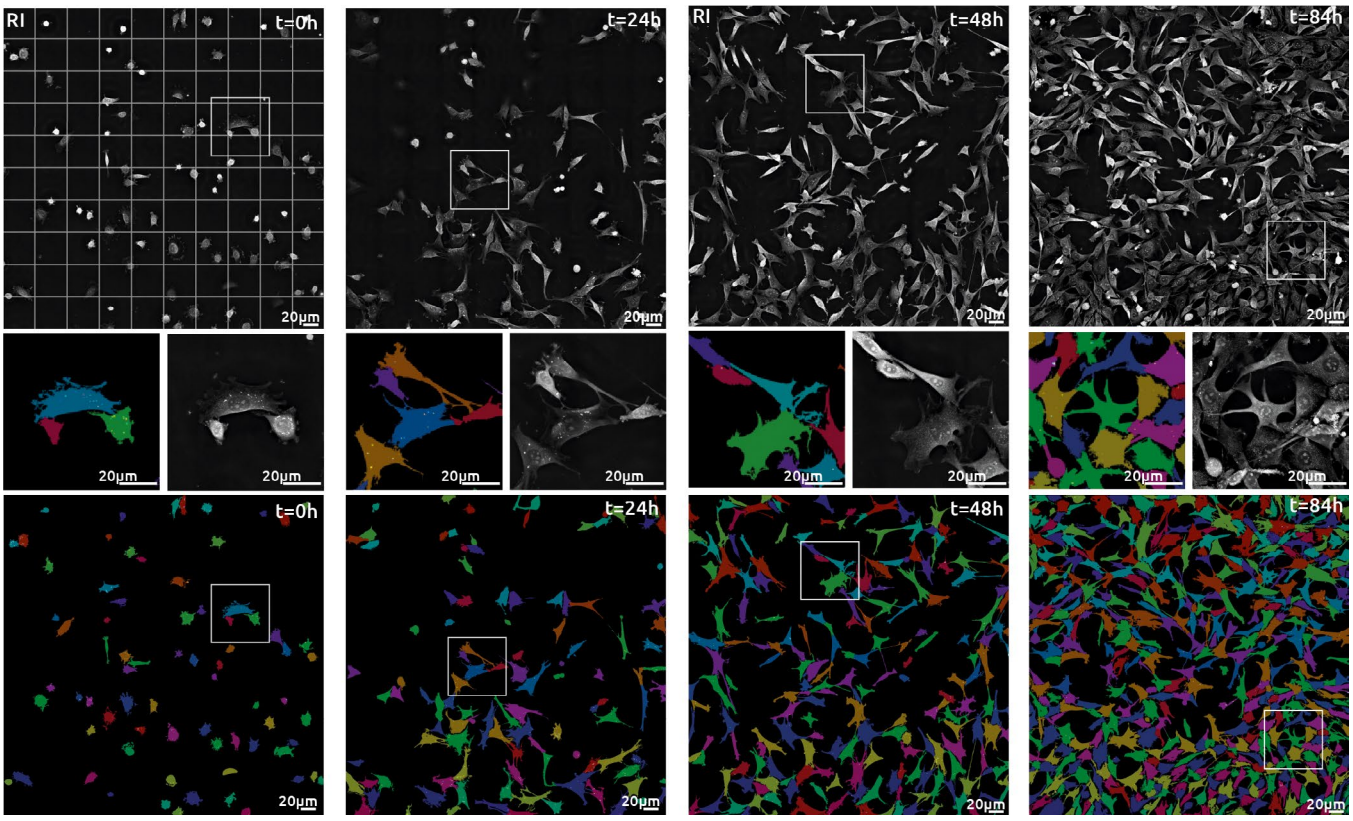
SHOWCASING EVE ANALYTICS

To test the efficacity of our segmentation technique over long experimental acquisitions, we used EVE Analytics to analyze an unperturbed population of 3T3-derived pre-adipocyte cells over 90 hours. Images were captured using the 10x10 grid-scan mode on Nanolive's CX-A (one image taken every 8.5 mins).

The results are displayed below. The top row shows the refractive index (RI) images captured by the CX-A The grid overlayed on the RI image at time point t=0 represents the 10×10 grid scan acquisition, which is stitched using a proprietary algorithm. The bottom row shows the segmentation mask generated by EVE Analytics and the middle row shows close ups corresponding to the square on each image.



✓ EVE Analytics can segment numerous single cells over thousands of images with no change in quality, regardless of the level of cell crowding/confluence in the image.

✓ The zoomed-in images show the high-precision of the segmentation. The fine details of the cell membrane protrusions (filopodia and lamellopodia) are captured, while touching cells are accurately delineated, proving that there is no trade-off between scale and precision.

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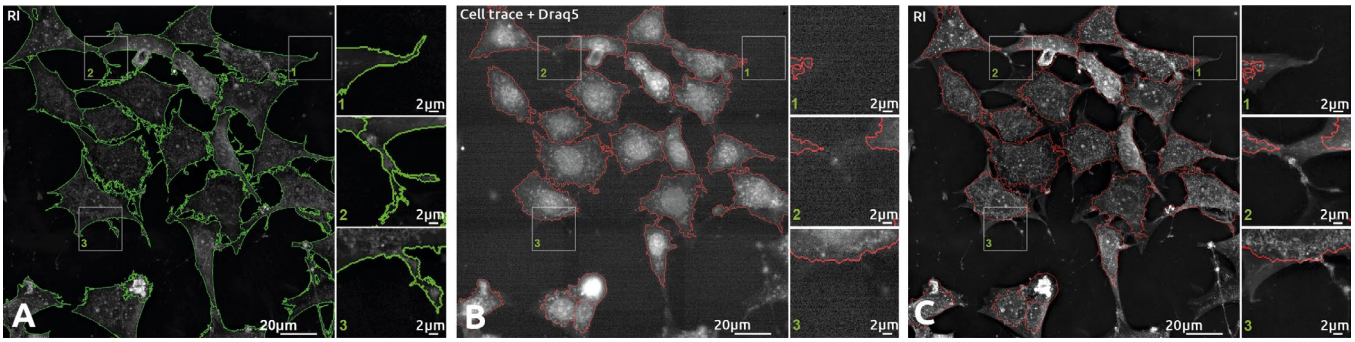
EVALUATING EA AGAINST FLUORESCENCE-BASED SEGMENTATION: METHOD

Direct comparison of EA against fluorescence-based segmentation can only be performed over short-term experiments, as cells die very quickly from phototoxicity when exposed to fluorescence. Nevertheless, we generated two datasets: one where 3T3-derived pre-adipocytes were exposed to low laser intensity (10%), and the other where they were exposed to high laser intensity (40%).

Condition name	Dyes	Laser intensity	Exposure (ms)	Imaging regime	Duration (h)
Low D+C	Draq5 (1:4000 dilution) Cell traceviolet (1:1000 dilution)	10%	200	1 RI and 1 fluorescent image every 4 mins	2
High D+C		40%			1

In both experiments, cells were stained with DRAQ5™: a modified anthraquinone that easily permeates the cell and interacts with double stranded DNA through weak stacking and hydrogen bonding, and CellTrace Violet™: an improved carboxyfluorescein succinimidyl ester (CFSE) based dye that readily diffuses into cells that is processed by esterases. Staining lasted 15 mins, and cells were washed 30 mins before acquisition. Refractive index (RI) images and fluorescence images were acquired simultaneously. Cells were segmented in EA and using the best Cell Profiler 4 (CP4) pipeline we could develop based on the fluorescence signal alone.

RESULTS: EVE ANALYTICS VS. LOW D+C



A. Cell segmentations computed using the RI signal only (using EA). **B**. Cell segmentations computed using the fluorescence signal alone. **C**. Segmentation outlines in B overlaid on the RI signal for comparison of segmentation performance.

Overlaying the fluo-based outlines over the RI signal (C) shows how many cellular details are missed using a live fluorescent imaging approach.

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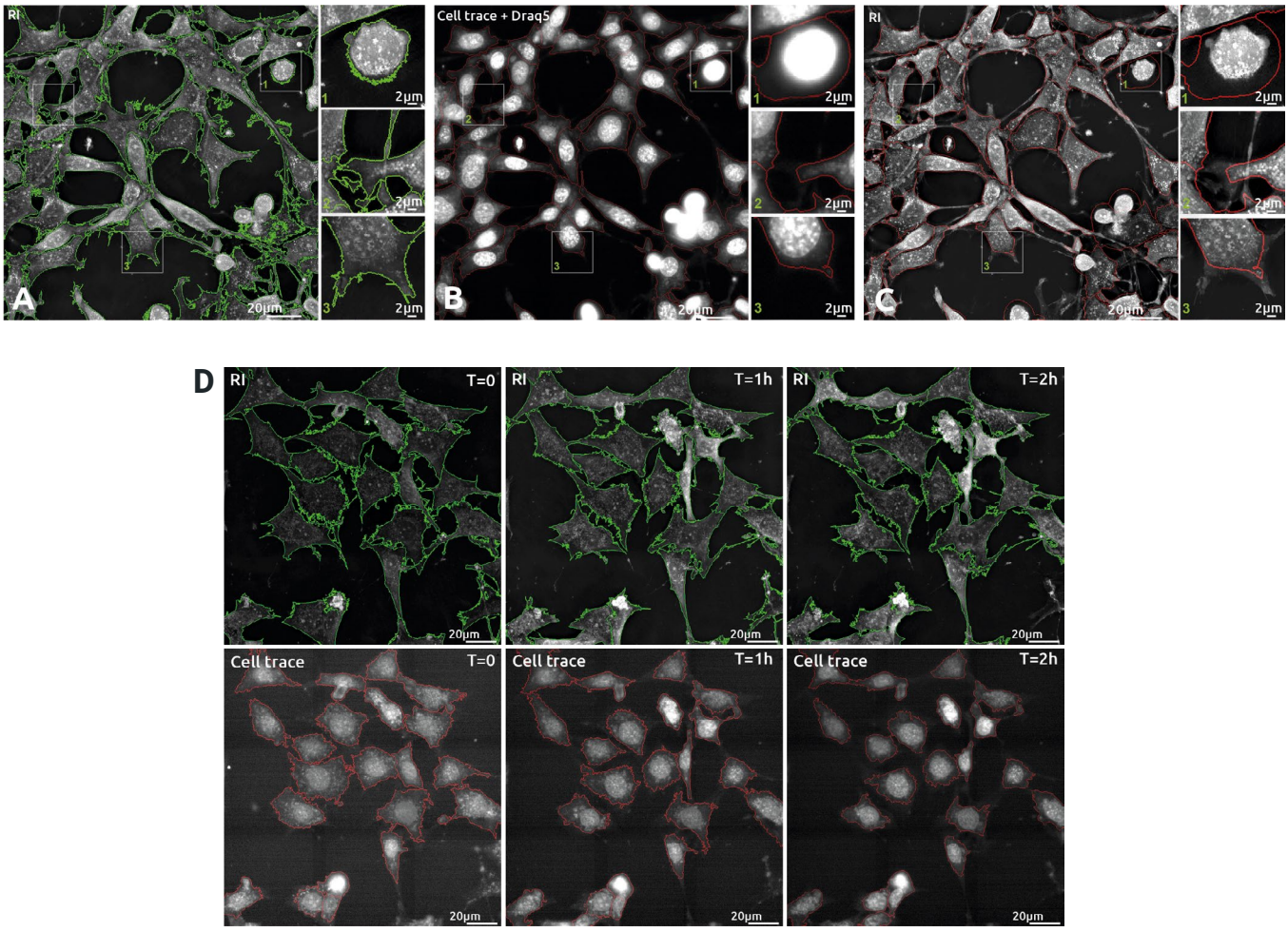
RESULTS: EVE ANALYTICS VS. HIGH D+C



A. Cell segmentations computed using the RI signal only (using EA). **B**. Cell segmentations computed using the fluorescence signal alone. **C**. Segmentation outlines in B overlaid on the RI signal for comparison of segmentation performance. **D**. Rapid deteriorations in cell health is the cost of generating a stronger fluorescent signal for improved segmentation performance.

Enhancing laser intensity provides one means of increasing the signal strength that is used for the segmentation (B). However, even with a stronger signal, EA consistently outperforms fluorescent-based segmentation, while the gain in fluorescent signal quality comes at a significant cost to the experimental duration. (D) Cells in high D+C conditions all showed signs of stress after 1 h and were dead after 2 h.

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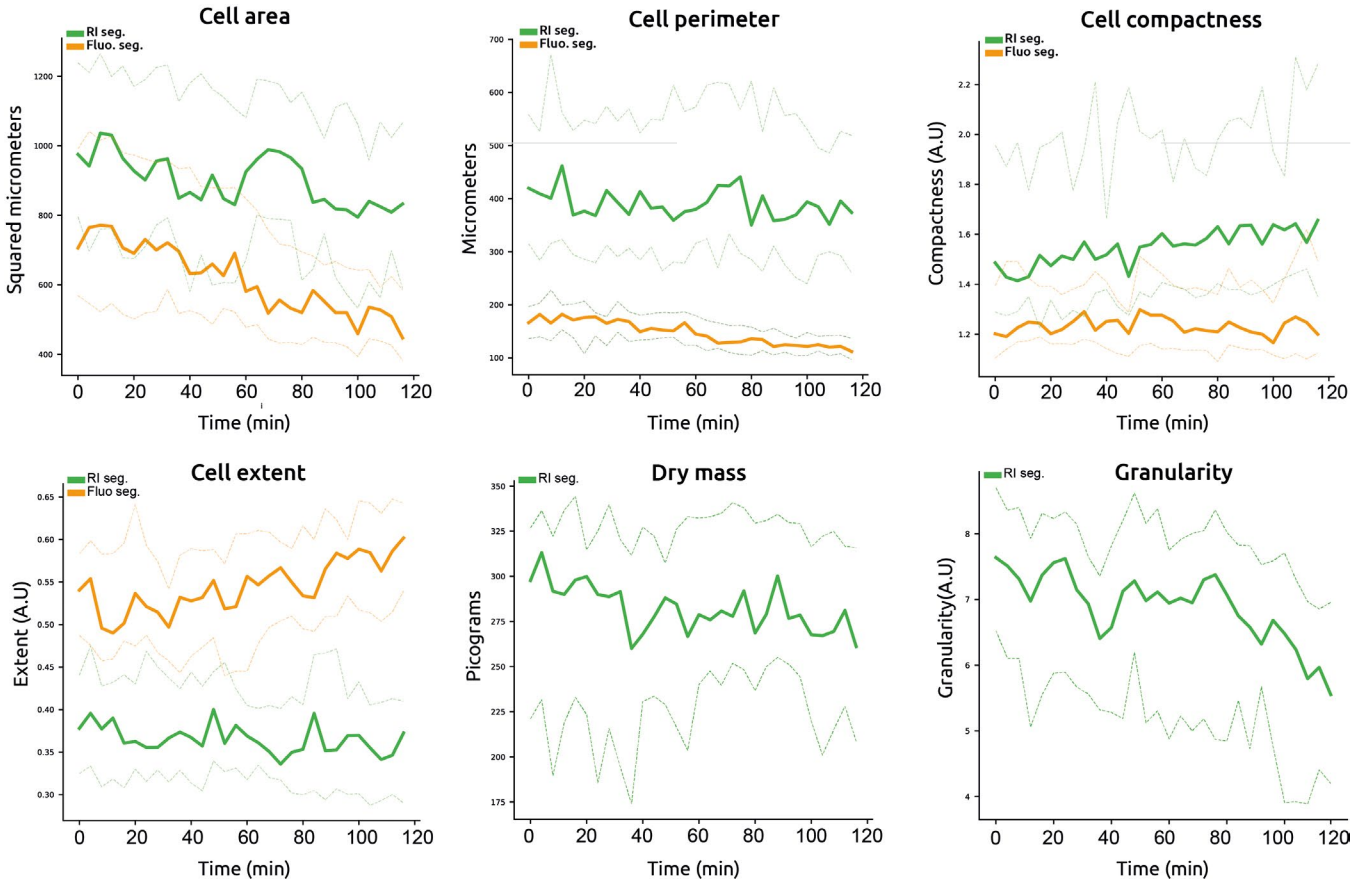
HOW ARE CELL METRICS IMPACTED?

EA automatically calculates numerous cell metrics. Here we compare cell area, perimeter, compactness, and extent calculated using EA (green) and compare them to the values generated by low D+C conditions (orange). In addition, we report one novel cell metric: dry mass, and exploit the full potential of a texture measurement called granularity, which are unique advantages of Nanolive imaging.



- The first striking observation is that the EA captures much greater cell-to-cell variability, as is demonstrated by the large interquartile spread of single cells values at each time point. This is of crucial importance as cell-to-cell variability reflects biological processes at work[10].
- The second fundamental observation concerns the inverse trends in compactness and extent. The values produced by the fluo-segmentation suggest cells are well spread (and by deduction healthy) but we know from the images that this is far from the case. In comparison, the values quantified using EA accurately reflect the stress the cells are experiencing.
- The final two plots represent metrics that are either inaccessible (dry mass) or that make little sense (granularity) using fluo-segmentations, further reinforcing the advantages of using EA.

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- ✓ Segmenting cells based on their refractive index signal is the best approach so far for quantifying live cell imaging experiments, both in terms of precision and non-invasiveness.
- ✓ EA works over long-term experiments where cell confluency changes significantly with no reduction in quality.
- ✓ The segmentations provided by EA only work using the RI signals obtained from Nanolive products and use proprietary strategies to reach precise cell segmentations over a broad variety of contexts.
- ✓ EVE Analytics delivers metrics that are inaccessible using fluo-segmentations, such as dry mass and exploits the full potential of a texture measurement called granularity.

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