Development of a 4-sample version of the Kolmogorov – Smirnov test for evaluating the temporal physiology of cells treated with test compounds in a label-free, high content, platform for quantitative analysis of adherent cell-culture models.

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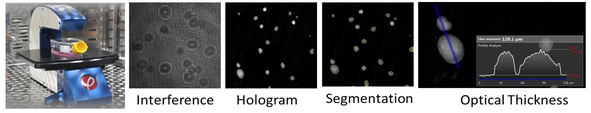
Quantitative Holographic Imaging Cytometry



- We employed a newly developed holographic imaging cytometry system HoloMonitor® M4 for label-free time-lapse cellular analysis (Phase Holographic Imaging, Sweden).
- Label-Free Imaging. Low power laser holographic imaging provides time lapse images of optical thickness.
- Segmentation routines are used to calculate cellular features for high content cellular analysis. This includes morphology, texture, motility, and many other features.
- In previous work we have demonstrated our ability to obtain quantitative, high content cellular feature data congruent to data obtained from traditional label-based systems.

Four Dimensional Imaging

- Four-dimensional imaging is an innovation that we developed and is perhaps the most informative venue for displaying phase holographic time lapse image data sets.
- The X position and Y positions, are the first two dimensions, the Z direction shows time, and the optical thickness of the X Y location is coded as the brightness (or color).
- Mitotic cells exhibit higher optical thickness, but not exclusively.
- This is analogous to confocal imaging stacks, with the difference being that the fundamental element is not a voxel (a concatenation of volume and pixel), but it is time related.

The Kolmogorov-Smirnov two-sample test

- The classic test takes control and test frequency distributions (histograms), and converts them to probability functions.
- The maximum vertical displacement between the two is reported as the D-value, to determine if the two distributions are significantly different.
- In cytometry applications, the test often reported false positive estimates of the significance.

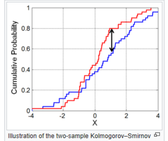


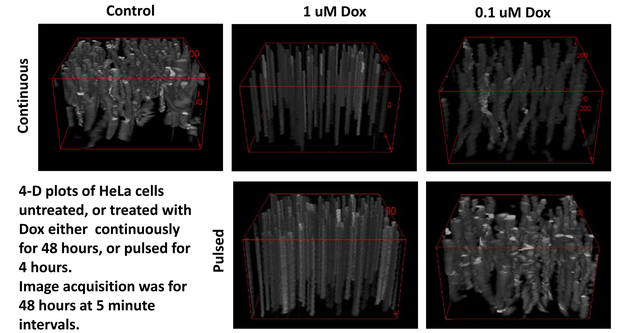
Illustration of the two-sample Kolmogorov–Sminov statistic. Red and blue lines each correspond to an empirical distribution function, and the black arrow is the two-sample KS statistic.

Image from Wikipedia

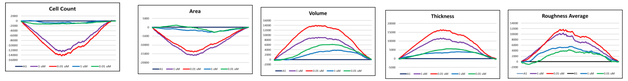
The modified Kolmogorov-Smirnov two-sample test

- The modified (comparator) version of the K.S. test has been in use in label based cytometry studies for decades.
- Instead of a single D-Value, histograms of the D-values are obtained.
- These histograms are termed Brownian Bridges, where the end points are fixed, and the function is free to vary in between.
- In our label free studies, we use the passage of time as the abscissa of the plots.

Continuous vs. pulsed Doxorubicin treatment of HeLa cells

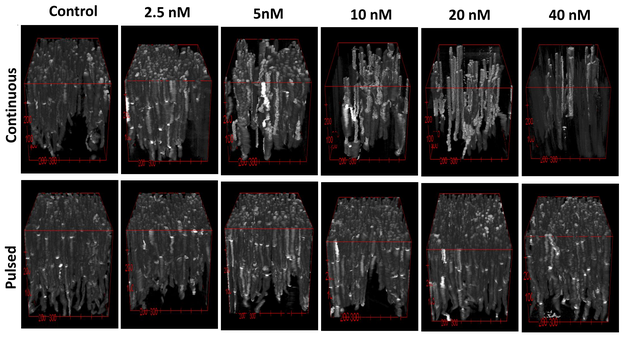


4-D plots of HeLa cells untreated, or treated with Dox either continuously for 48 hours, or pulsed for 4 hours.
Image acquisition was for 48 hours at 5 minute intervals.

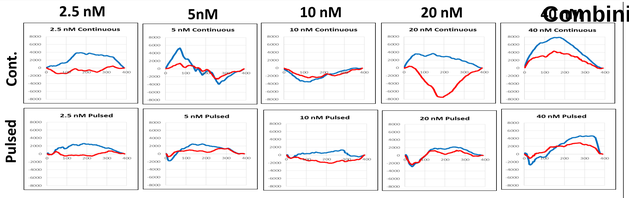


Brownian Bridge plots for the indicated features. Color coding is control (black), 1 uM C. (purple), 1 uM P. (red), 0.01 uM C. (blue) and 0.1 uM P. (green).

Continuous vs. pulsed PCT treatment of HeLa cells



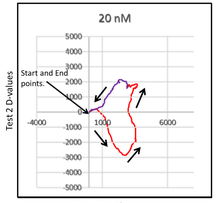
- 4-D plots of HeLa cells untreated, or treated with Paclitaxel (PCT) at the indicated concentrations, either continuously for 36 hours, or pulsed for 4 hours, and then washed and imaged.
- The mechanism of PCT toxicity is that the microtubules of cells are polymerized and cells are prevented from completing mitosis. Eventually, cells will undergo apoptosis and then die.

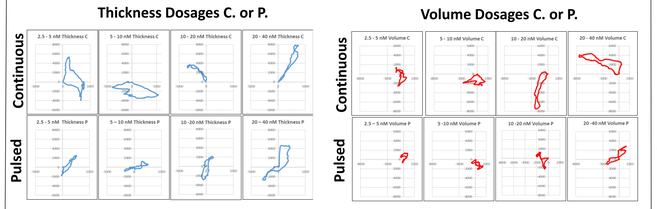


- Two parameter K.S. Brownian Bridge plots of the data derived from the Paclitaxel Continuous vs. Pulsed experiment.
- Blue = average cell thickness. Red = average cell volume.

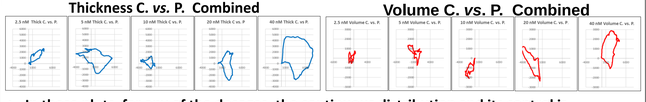
Combining 2 K.S. evaluations = four- sample method

- For each time point 2 values are plotted. The time points are processed chronologically with the points from the first 24 hours of the acquisition colored red, the values from the second half colored purple.
- The displayed connected lines show the time course of the experiment (black arrows).
- *Like* the Brownian Bridge plots, the end points of the distributions are held at a constant value (0).
- *Unlike* the Brownian Bridge plots, the end points are at the same location in the plot
- A "Roller Coaster" analogy is more appropriate.



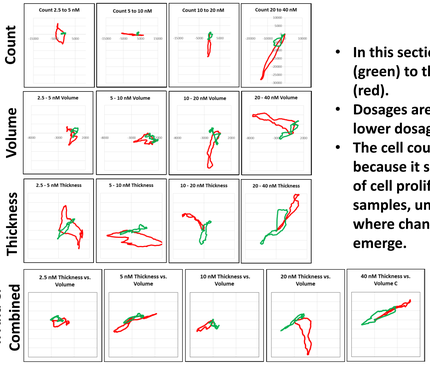


- In these 4 sample plots, the 2D result from a given concentration compared to its control (A *vs.* Ac) are compared to the next lower dosage (B *vs.* Bc).
- The bounded regions are the probability functions of the changes between the two dosages.



- In these plots, for any of the dosages, the continuous distribution and its control is compared to the pulsed treatment distribution and its control.

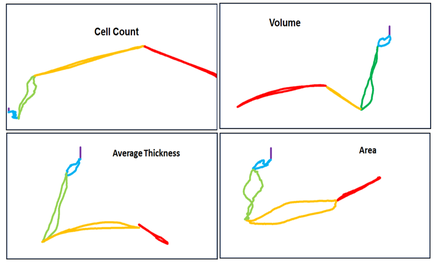
Combining 2 four sample evaluations = eight-sample method



- In this section, the pulsed samples (green) to the continuous samples (red).
- Dosages are compared to their next lower dosage.
- The cell count feature was added, because it shows that there is very loss of cell proliferation in the pulsed samples, until the highest dosages where changes are just starting to emerge.
- Here, the thickness and volume are combined at the various single dosages.

Comprehensive time evaluation of a Dox toxicity experiment.

- HeLa cells were treated with Dox at 0, 0.01, 0.1, 1, 10, and 100 u.
- Imaging was for 48 hours at 5 minute intervals.
- 4P K.S. tests were performed for each dosage compared to the next lower dosage.
- Resultant probability vectors were plotted in head to tail fashion (violet, blue, green, yellow, red) in increasing dosage.



- The length, breadth and directionality of the vectors is proportional to the amount of change in the cell population between the given dosages.

Summary

- Recently, the HoloMonitorM4 holographic quantitative imaging system has become available as a label free alternative to fluorescence analysis in high content cellular analysis.
- The system allows for long term imaging of multiple samples.
- We developed 4-dimensional imaging plots of holographic images over time to assist in the visualization of the effects of pharmaceutical compounds under development.
- We found that we could use the same analytical techniques that we were implementing in our fluorescence based analyses, including a modified Kolmogorov-Smirnov 2 sample being used as a comparator.
- We developed extended functionalities for the comparative K.S. tests to allow tracking the changes in cellular populations over long periods of time.