



A label-free, high content, moderate throughput analytical platform for quantitative kinetic analysis of cell behavior upon drug activation based on the Kolmogorov-Smirnov test.



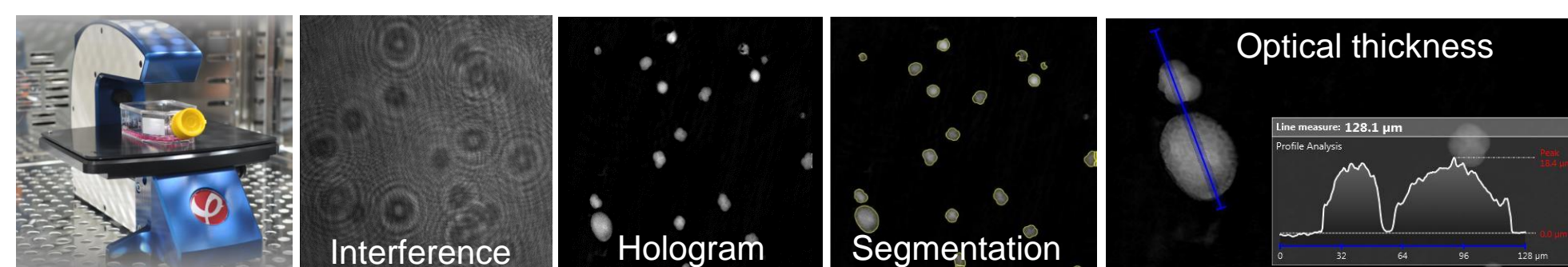
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Introduction

Our objective is to develop multi-functional nanotechnology-based anti-tumor drug delivery systems for improving efficacy of treatments and reducing undesirable side effects. The essential part of this process is the development of un-biased quantitative analytical techniques. We are reporting a successful validation of a very high content, medium throughput system in multi-well plates.

Holographic Imaging Cytometry



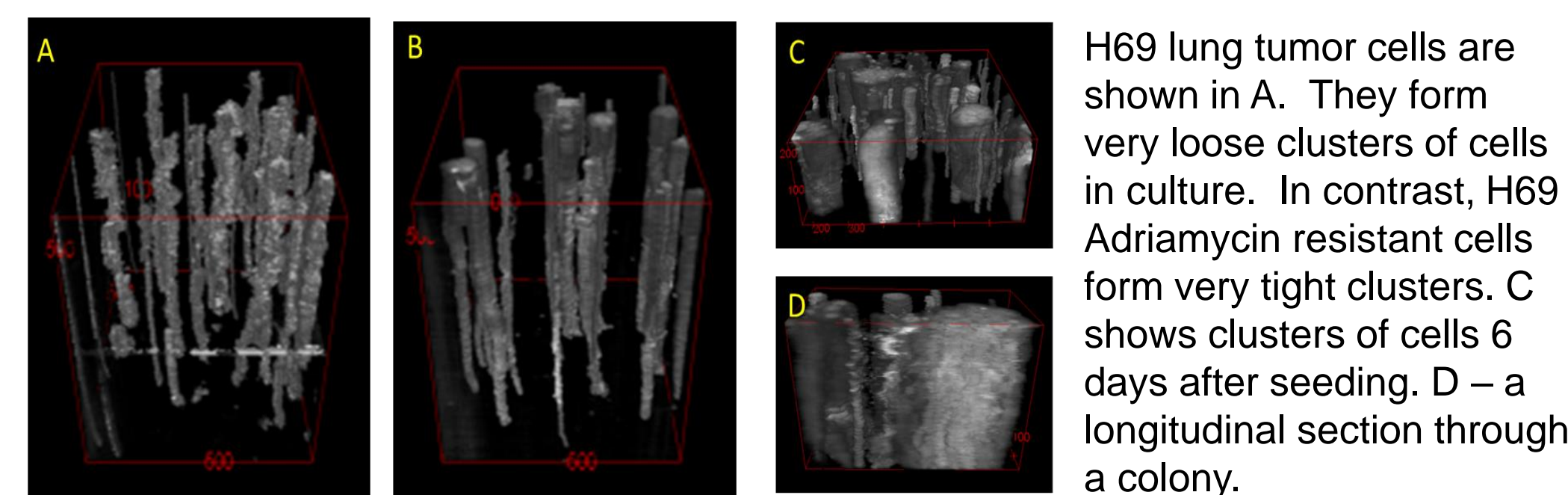
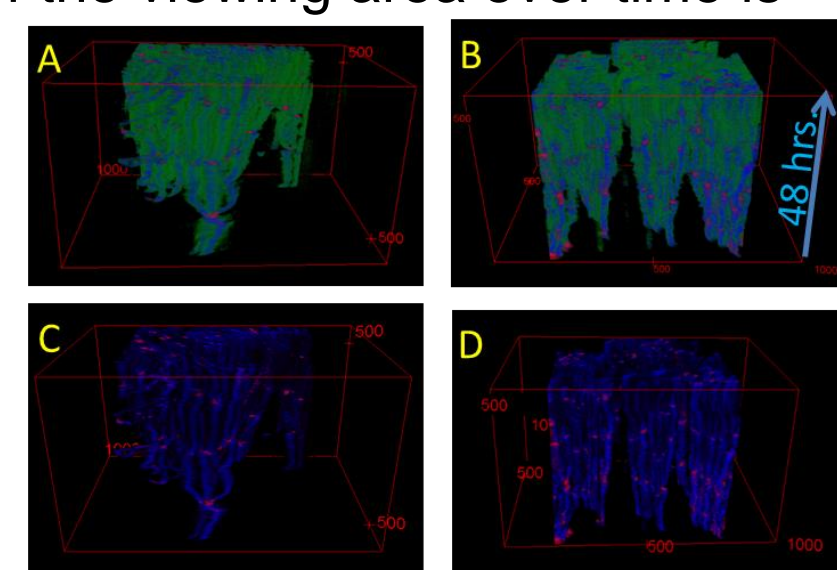
We employed a newly developed holographic imaging cytometer HoloMonitor® M4 for label-free time-lapse cellular analysis (Phase Holographic Imaging, Sweden), typically at 5 minute intervals for 48 – 72 hours. A low power red laser is split into sample and reference beams to obtain holograms of cells. The holograms are unwrapped by proprietary software into quantitative dark field images, with very precise calculation of the cell optical thickness. These images are segmented, and many features are extracted for cellular events. In our evaluations we found that cell optical thickness, volume and area have high correlation with features used in traditional fluorescent DNA-stained analysis. We obtain full cell cycle profiles, including the separation of mitotic cells – all in a label free environment.

An automated stage became available allowing acquisition of multiple fields in each time point. We are now using 6-, 12-, and 24-well microtiter plates which significantly enhance our experimental protocol design options and increases throughput.

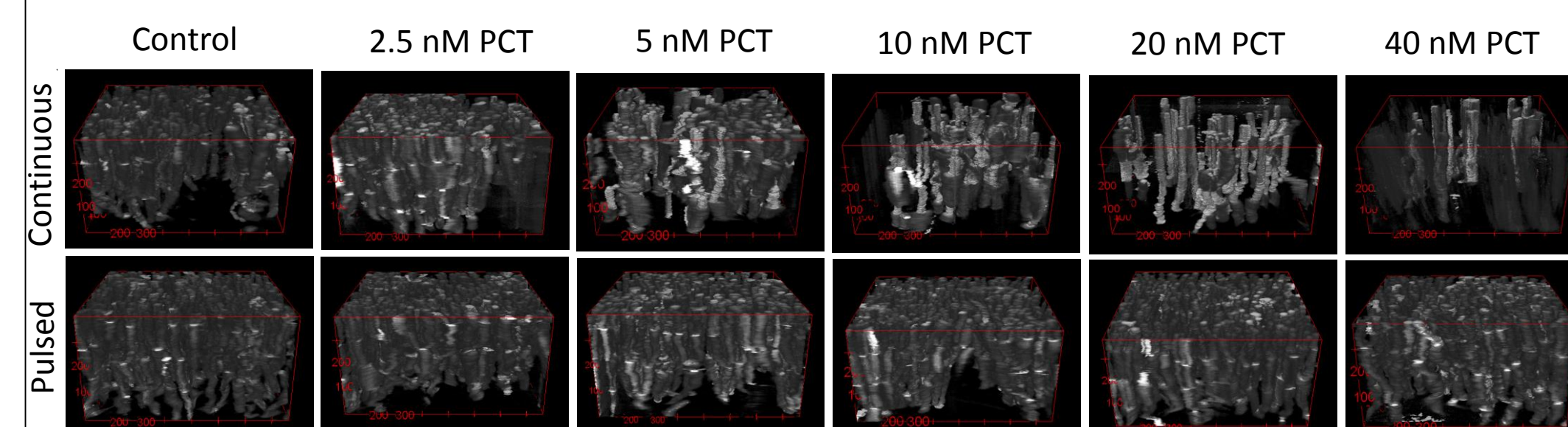
4D Visualization

We developed a novel 4D image display of evaluated fields of view: X position, Y position, cell thickness coded as brightness over time in the Z direction. In these images, the history of the viewing area over time is displayed.

HeLa cells from the ATCC (A, C) are compared to a tumorigenic strain (B, D). Coloration is based on optical thickness (green = low, blue = intermediate, red = high). The red spots are mitotic cells. The tumorigenic cells form tighter clusters.



H69 lung tumor cells are shown in A. They form very loose clusters of cells in culture. In contrast, H69 Adriamycin resistant cells form very tight clusters. C shows clusters of cells 6 days after seeding. D – a longitudinal section through a colony.



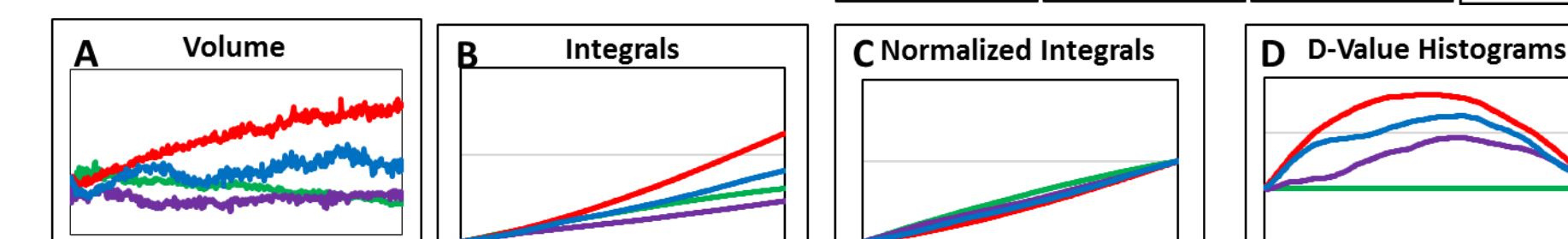
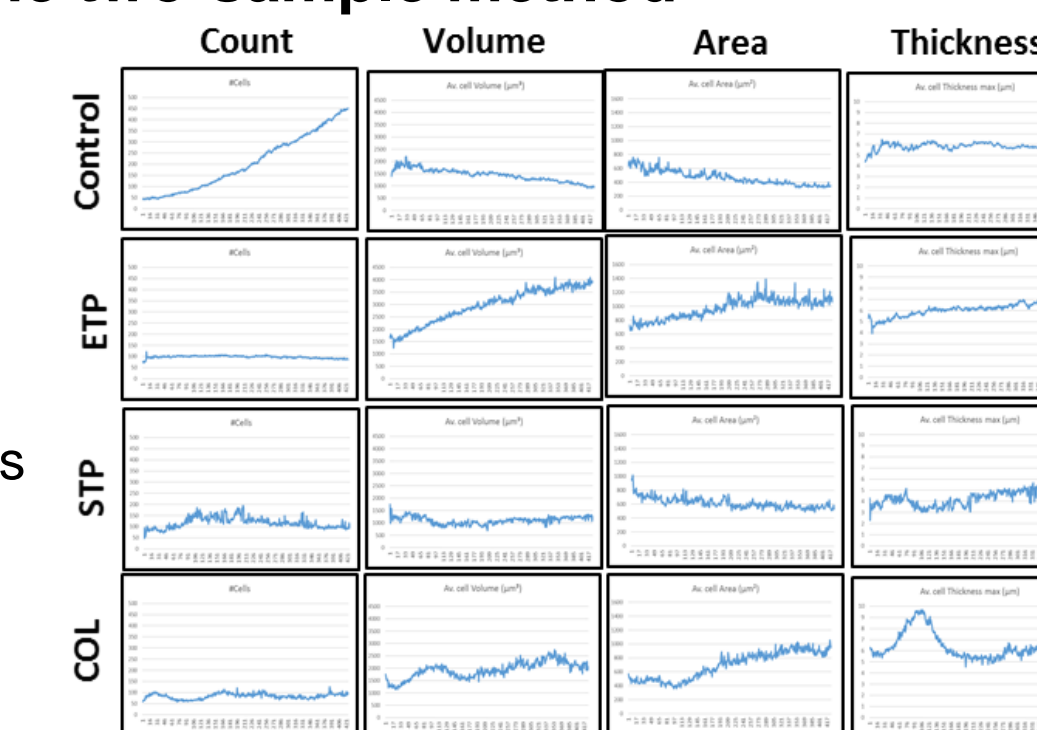
HeLa cells were treated with the indicated dosages of paclitaxel (PCT), either with continuous exposure, or pulsed for 4 hours followed by 48 hours of imaging.

The modified Kolmogorov-Smirnov two-sample test

- The test allows evaluating a continuous probability distribution to determine if it **significantly** conforms to a mathematical function such as a Gaussian distribution (one sample test) or if two distributions are **significantly** different (two sample test).
- Functionally, the test creates what is termed a “Brownian Bridge” – a probability function where the two end points are at fixed points, and the intervening points are allowed to fluctuate. The amount of fluctuation is the metric of the test.
- It has a long history of usage in Quantitative Analytical Cytometry, both in flow based and image based platforms using fluorescent dyes as a comparator.
- In our studies, we are interested in showing the amount of difference between treated samples, and their appropriate controls.

Example of the two-sample method

L929 cells were untreated, or treated with Etoposide, Staurosporine, or Colcimide and imaged for 72 hours at 10 minute intervals. At right is an array of plots of selected features over time, shown on the X-axis.

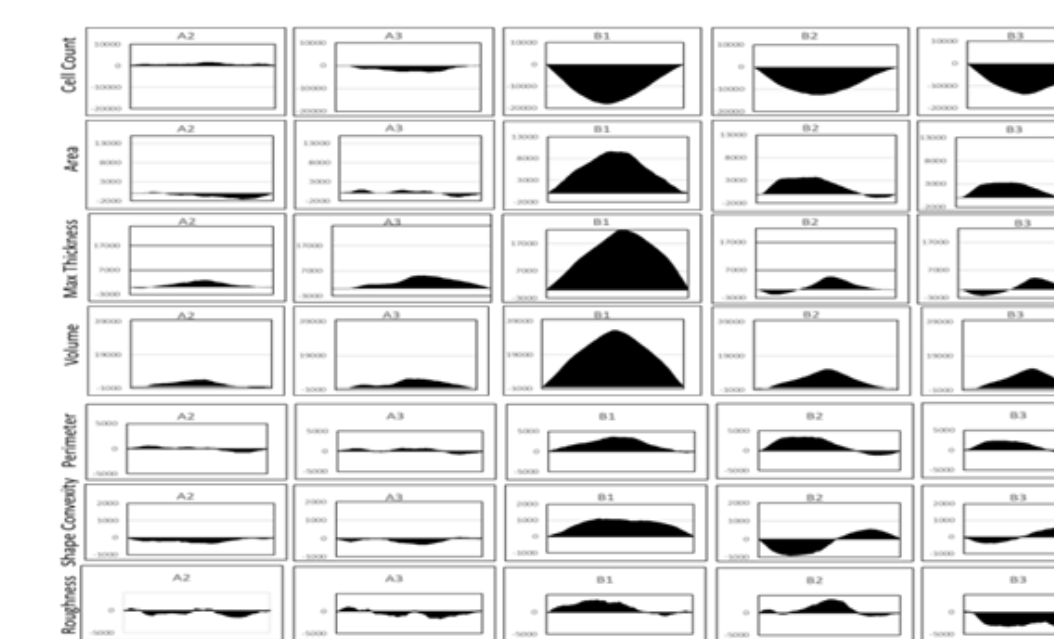


- A.** Continuous distributions are imported from the HoloMonitor M4 software.
- B.** The cumulative distributions are calculated – the integral of the distributions.
- C.** The integrals are normalized to obtain probability distributions.
- D.** The Normalized value for each sample is subtracted from its control to obtain “Brownian Bridges” – distributions with the end points fixed and the intervening values free to fluctuate.

HeLa cells exposed to different dosages of Doxorubicin

HeLa cells were treated with Doxorubicin at concentrations ranging from 1.6 nM to 1uM at 5 fold increments. Imaging was performed for 48 hours at 5 minute intervals.

Note that the plots can be up-going if a feature is greater than the control, or down-going if the feature is less than the control.

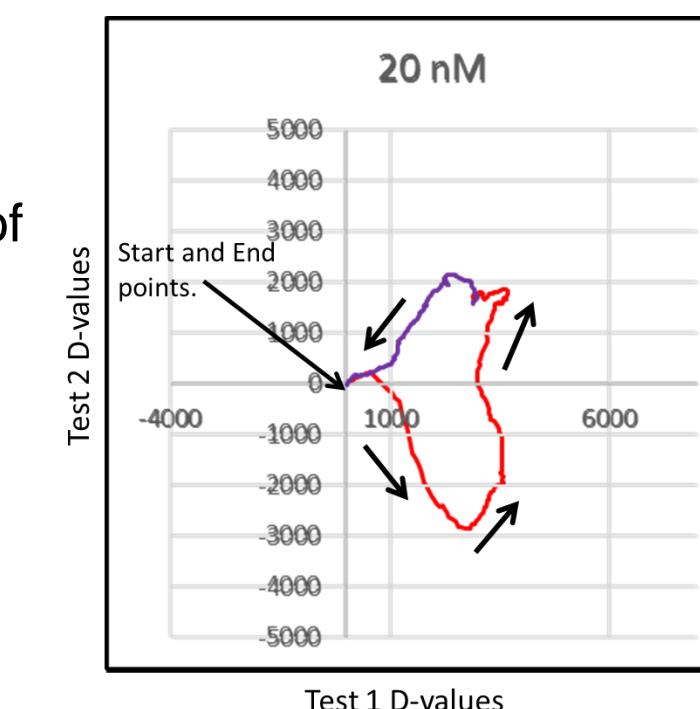


At low concentrations, unrepaired DNA strand breaks start to accumulate, and cause slight increases in the volume and thickness of the cells. At mid-dosage, the DNA repair process is overwhelmed, proliferation is halted, and there are vast changes in the morphology of the cells. At the higher concentrations, we see the changes in the morphology of the cells as they become senescent, die and eventually deteriorate.

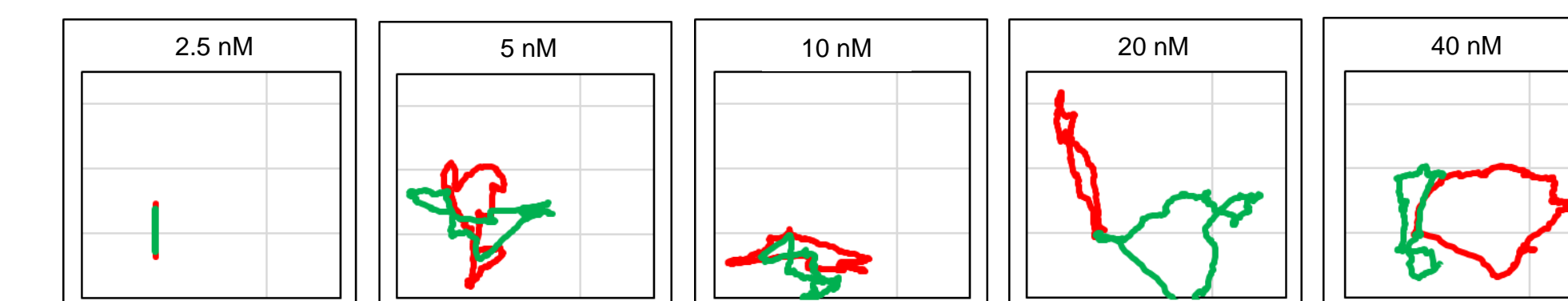
We are using this methodology to systematically evaluate all of the extracted features from the HoloMonitorM4 software (approximately 40) to determine which ones have the most biological relevance, and under which conditions.

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

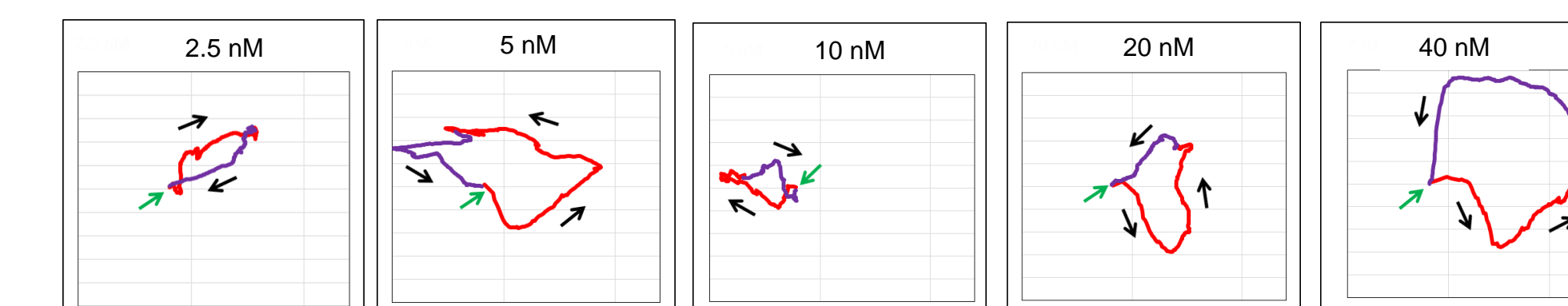
- For each time point 2 values are plotted. The time points are processed chronologically with the points from the first 24 hours of the acquisition colored red, the values from the second half colored purple.
- The displayed connected lines show the time course of the experiment (black arrows).
- Like 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. People board and disembark at the same point, but during the ride there is complete freedom of motion.



Continuous vs. pulsed PCT treatment of HeLa cells



Comparing two methods at different treatment levels (HeLa cells continuously exposed to Taxol for 52 hours = RED; pulsed Taxol exposure for 4 hours followed by 48 hr. imaging = GREEN). Each dosage was compared to the immediately lower dosage. The average cell volume is plotted. By the 20 nM dosage, the two populations are completely separated, with the “continuous” cells all being dead, but the “pulsed” cells are still alive.



Comparisons of continuous to pulsed treatments: Red = the first 26 hours, Purple = the last 26 hours. Green arrow points to the start. Black arrows show time passage. We are following the complex pharmaco-dynamics at the different drug concentrations.

Summary

- The HoloMonitorM4 holographic quantitative imaging has proven to be well suited for our evaluations enabling label-free kinetic high content analysis on a per cell level.
- 4-dimensional imaging plots of holographic images over time (with cell thickness being particularly useful) permit evaluation of drug effects in the kinetic context of the entire experiment.
- A modified Kolmogorov-Smirnov test was applied to compare treated samples to their controls.
- Our newly developed 4-sample variation of the test allows quantitative analysis of the population changes over the entire (kinetic) evaluation time.