Comparison of the effects of pharmaceutical compounds on tumor cells in 2D and 3D *in vitro* models using label-free, quantitative 4 dimensional holographic imaging.

Ed Luther[1], Giuseppina Salzano[2], Shravan Kumar Sriraman[2] , Daniel Costa[2,3] and Vladimir P. Torchilin[1,2,4]

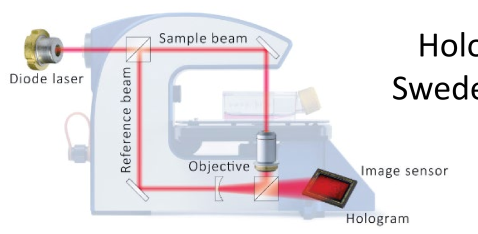
Department of Pharmaceutical Sciences, Northeastern University[1], Center for Pharmaceutical Biotechnology and Nano Medicine[2], Northeastern University, Boston MA, Department of Biochemistry, Faculty of Science, King Abdul-Aziz University, Saudi Arabia[3], CAPES Foundation, Ministry of Education of Brazil, Brasília, Brazil[4]. Corresponding author **Ed Luther e.luther@neu.edu**.

Introduction

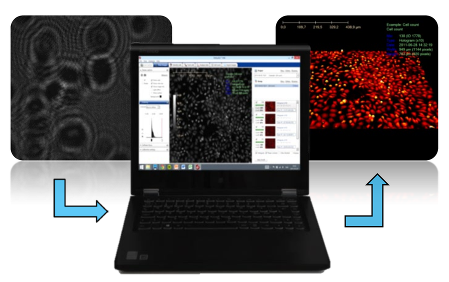
Development of *in vitro* models for the evaluation of drugs represents a useful approach as *in vivo* studies may be costly and time consuming. Ideal models should take into account the effects of the cellular microenvironment, which includes the extra-cellular matrix, stroma and neighboring cells.

Holographic Imaging Cytometry

Instrumentation:

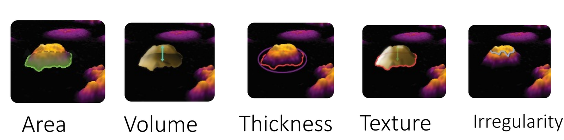


Time-lapse holographic imaging cytometer HoloMonitor® M4 (Phase Holographic Imaging, Lund, Sweden) for label-free long-term kinetic cellular analysis.



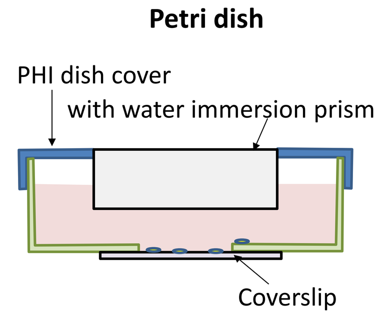
- Holographic imaging of interference patterns from a low power 635 nm diode laser.
- Multiple segmentation algorithms to identify objects.
- XY co-ordinates enable cell tracking over time.
- Time-lapse acquisition at selected intervals ranging from seconds to multiple days.
- Automated stage for multiple sample acquisitions.
- Operable inside tissue culture incubators.

Quantitative phase shift measurements are translated by software algorithms into morphological parameters:



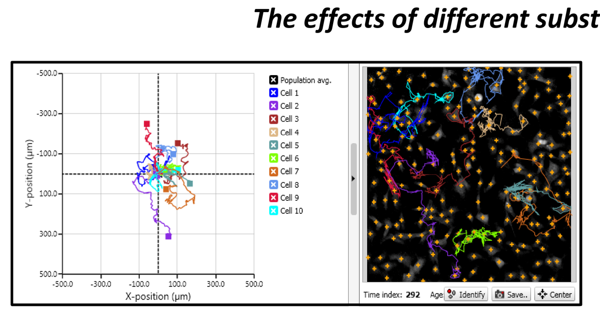
Experimental protocols:

- Experiments were performed on glass-bottom Petri dishes (MatTek, Ashland MA).
- A proprietary dish cover (Phase Holographic Imaging) with a water immersion prism was employed to mitigate the effects of condensation and vibrations.
- In some experiments, the collagen and poly-L lysine treated dishes were used.

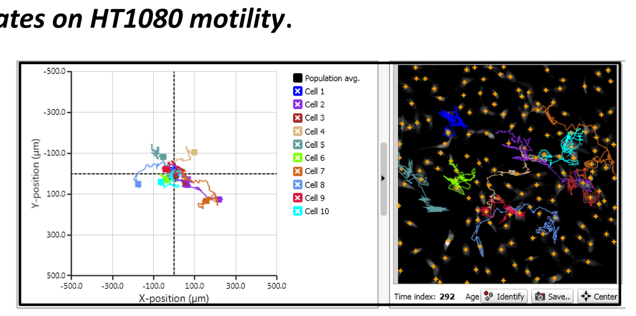


- HeLa (human cervical adenocarcinoma) and HT-1080 (human fibroscarcoma) cells were obtained from the ATCC.
- In 2D studies, medium was used; cells would rapidly settle to the bottom surface and adhere and acclimate for 24 hours. Cultures were treated with test compounds for 4 hours, the media was changed, and long-term imaging followed.
- In 3D studies, cells were first allowed to adhere to the bottom surface and treated with test compounds for 4 hours. The media was removed and replaced with 1 mg/ml collagen containing media.

The effects of different substrates on HT1080 motility.



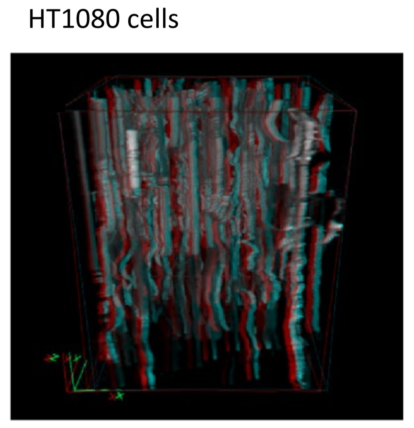
Poly-L lysine coated Petri dishes promote cell motility



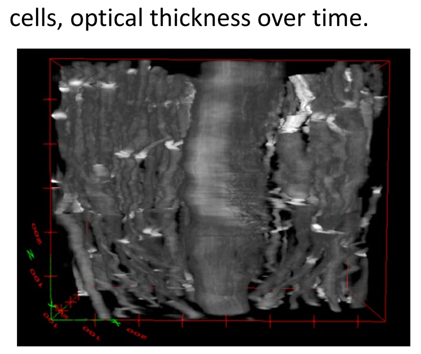
Collagen substrate inhibits motility of HT1080 cells

4D image generation using Image J software:

- 2Dimensional images of each time point were exported into NIH ImageJ software.
- Images were converted to a stack.
- The stack was processed with the 3D viewer plugin.
- Stereoscopic renderings were obtained by rotating a 3D projection slightly in the +X and –X directions and processing the images in Sterophotomaker software.
- 4D image stacks can be optically sectioned to reveal the internal optical structure.



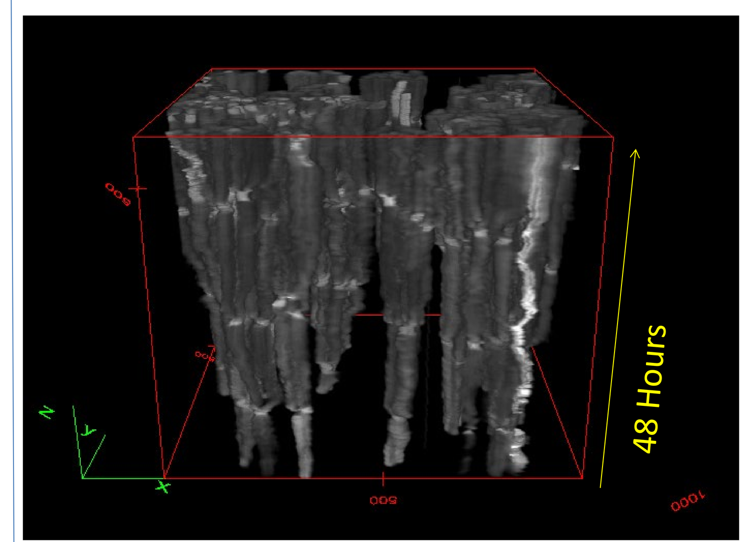
Anaglyphic projections of the field of view showing the X and Y position of cells, optical thickness over time.



Optical section through the center of a giant HeLa cell and the neighboring normal cells.

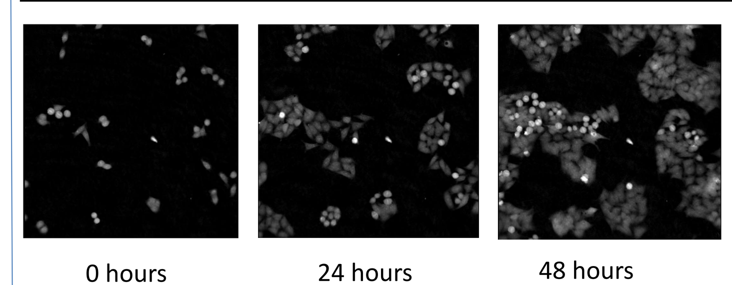
2D models of non-motile adherent cells

X-position, Y-position and Optical Thickness of Untreated HeLa cells over 48 hours



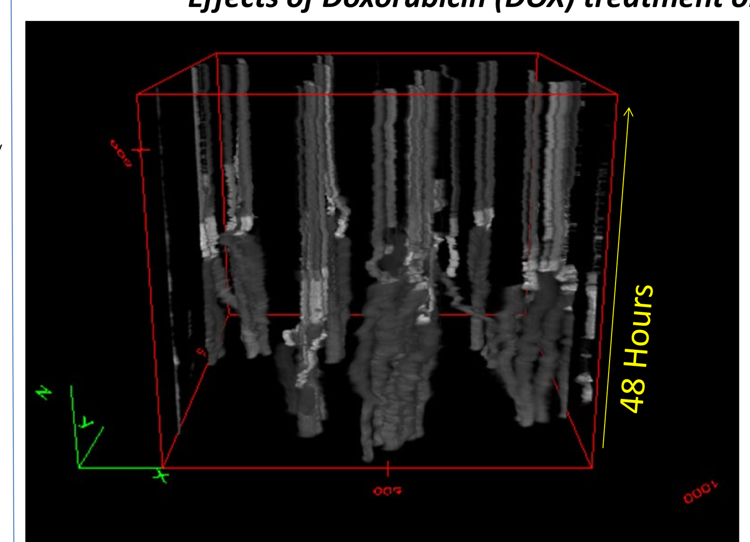
The white v-shaped spots are mitotic cells, with increased density as cells round up.

The bright tracks are cells undergoing mitotic dysfunction.



Hela cells are non-motile. Untreated colonies expand in size as a result of pressure exerted by the grow of the newly formed daughter cells.

Effects of Doxorubicin (DOX) treatment on HeLa cells over 48 hours

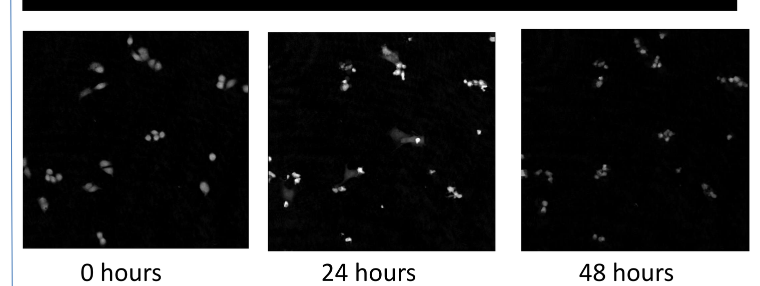


Hela cells were treated with a lethal dosage of free DOX.

The toxic effects first appear at around the same time point where cells in the culture enter mitosis.

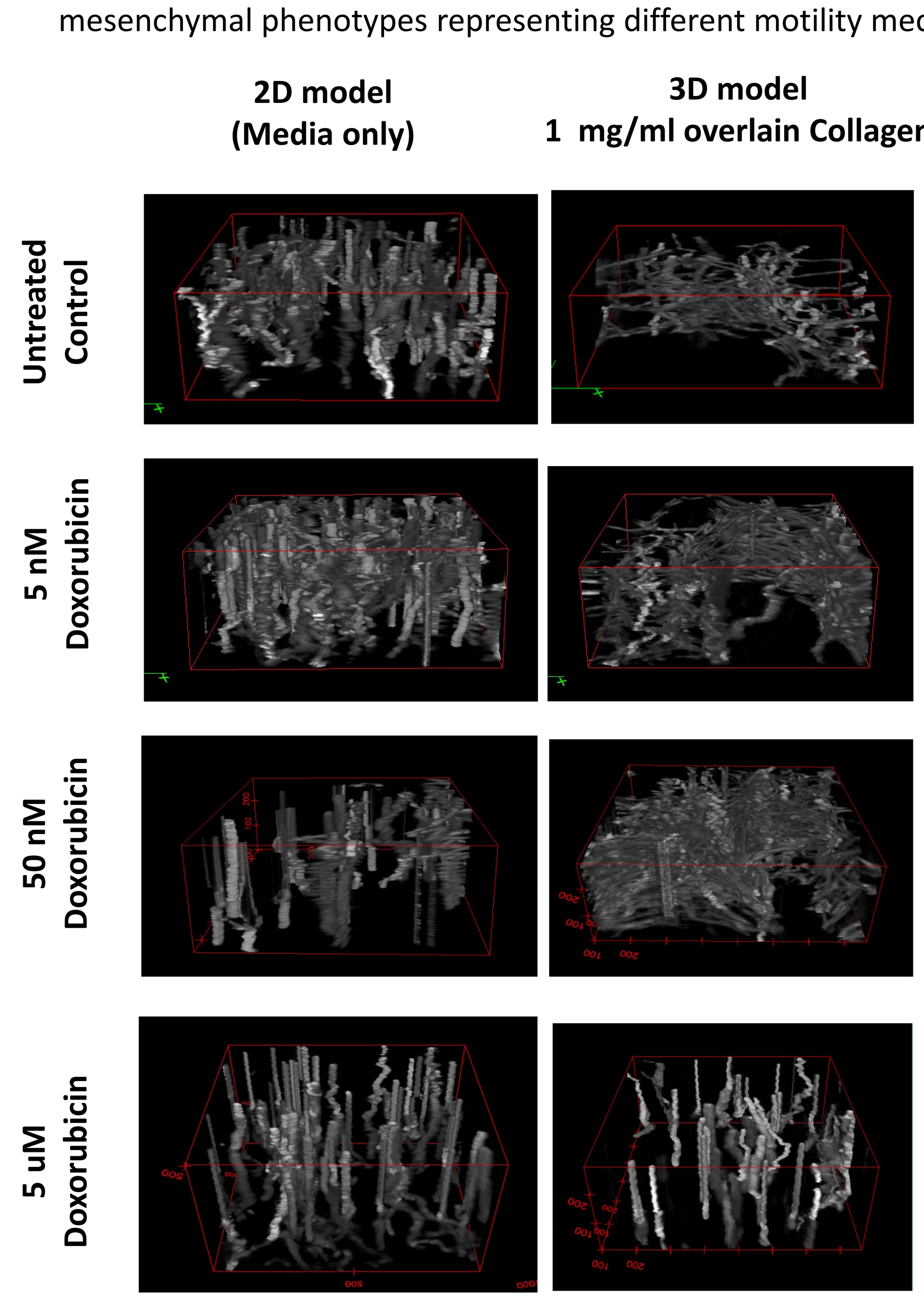
There is an increase in the density of cells (whiteness) as a result of pyknosis, evidenced by the irregular shapes of the cells.

Afterwards, the cells present as fading spires as the cells gradually disintegrate.



2D and 3D models of motile adherent cells

Human HT-1080 fibrosarcoma cells alternate between amoeboid and mesenchymal phenotypes representing different motility mechanisms.



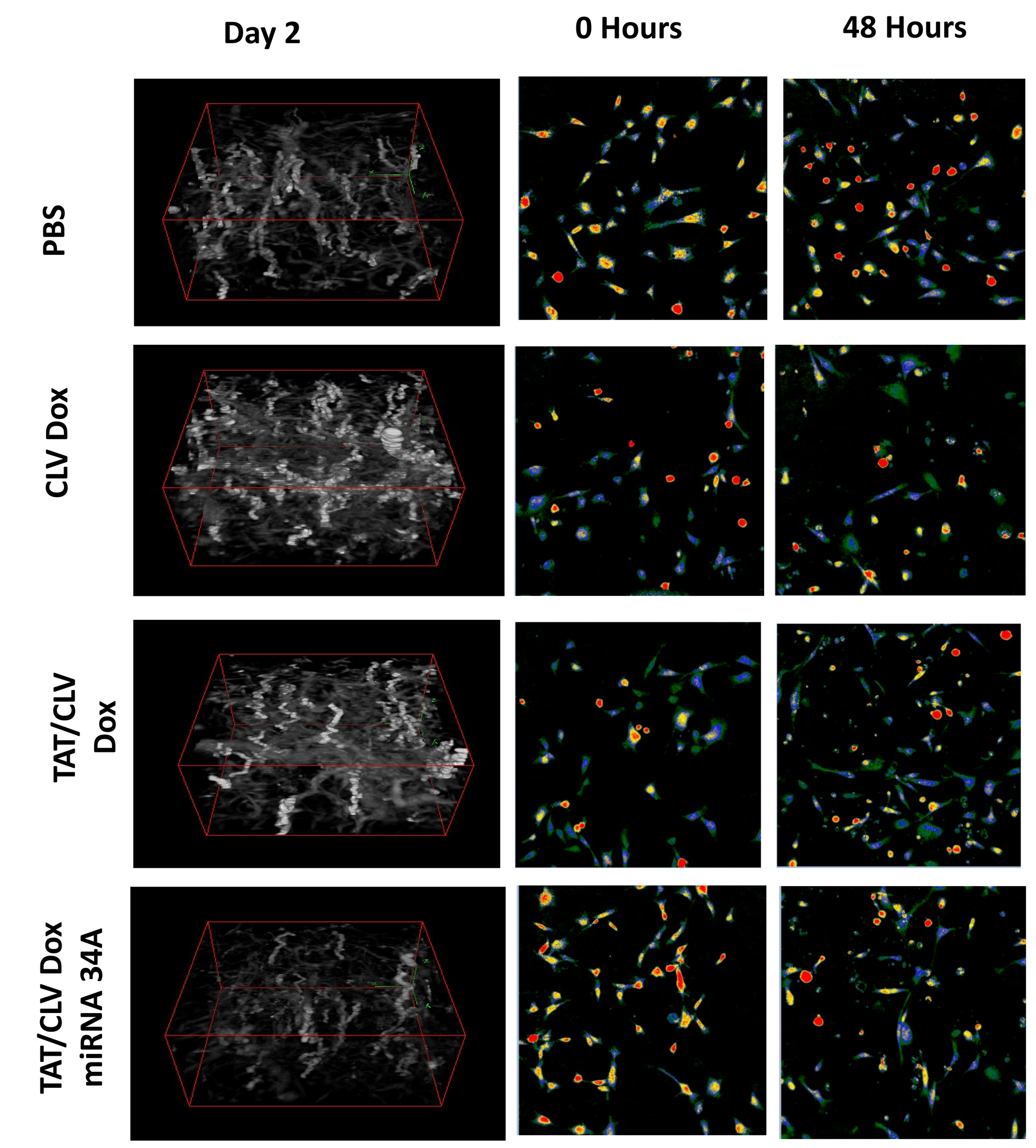
Low concentrations of DOX inhibit migratory motion in 2D cultures, but not in 3D culture models.

High concentrations of DOX inhibit cell motility and lead to cell death.

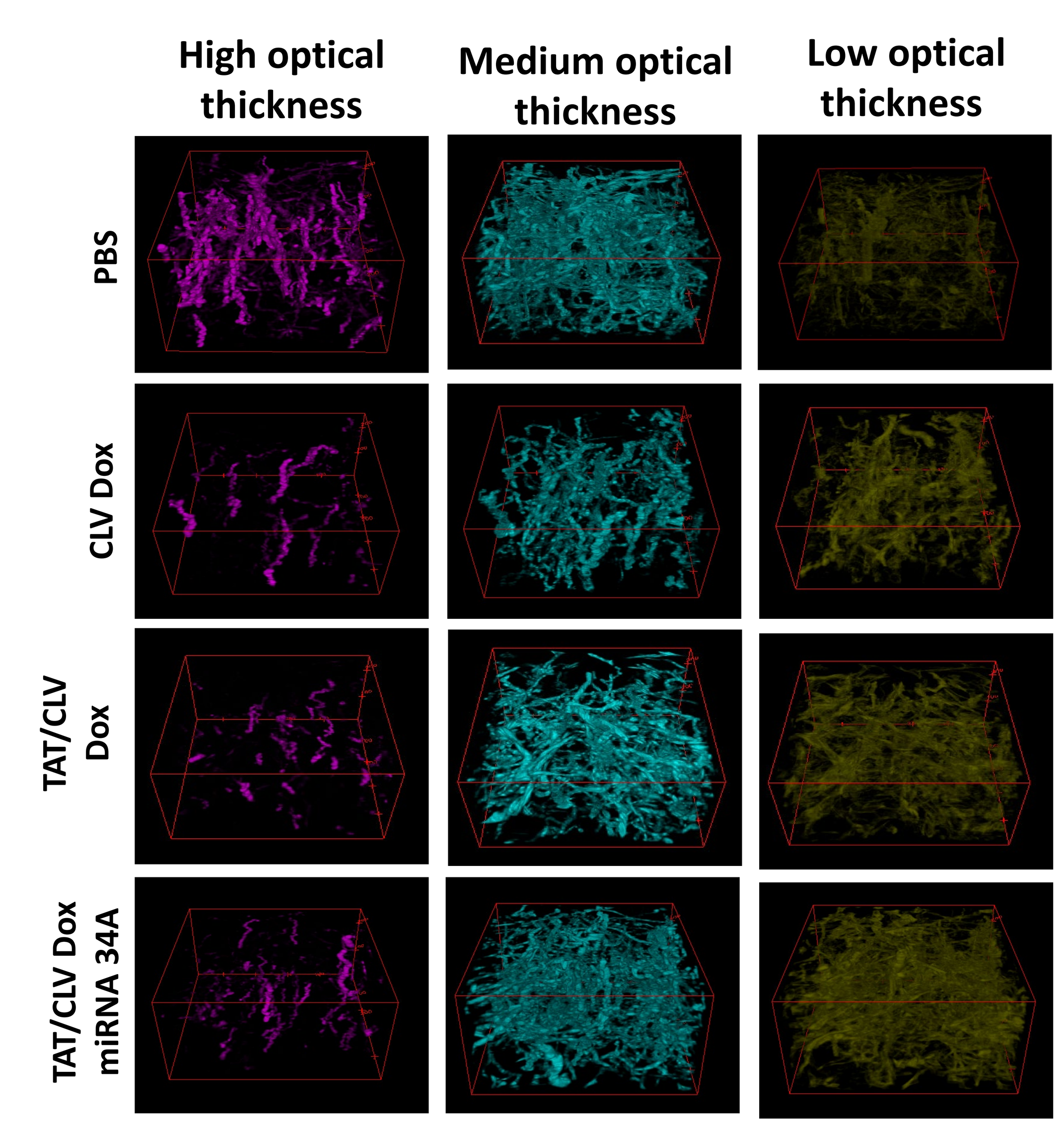
Pharmacodynamics application

We are developing multifunctional nanoparticles that specifically target tumor cells. The components include:

- CLV Dox Conjugate, which releases Doxorubicin after cleavage by MMP2 enzyme (overexpressed in HT-1080 cells).
- TAT conjugate, which helps in internalization of the particles by the cells.
- miRNA 34A conjugate, which down-regulates cell death regulatory proteins, such as Bcl-2 and Survivin.



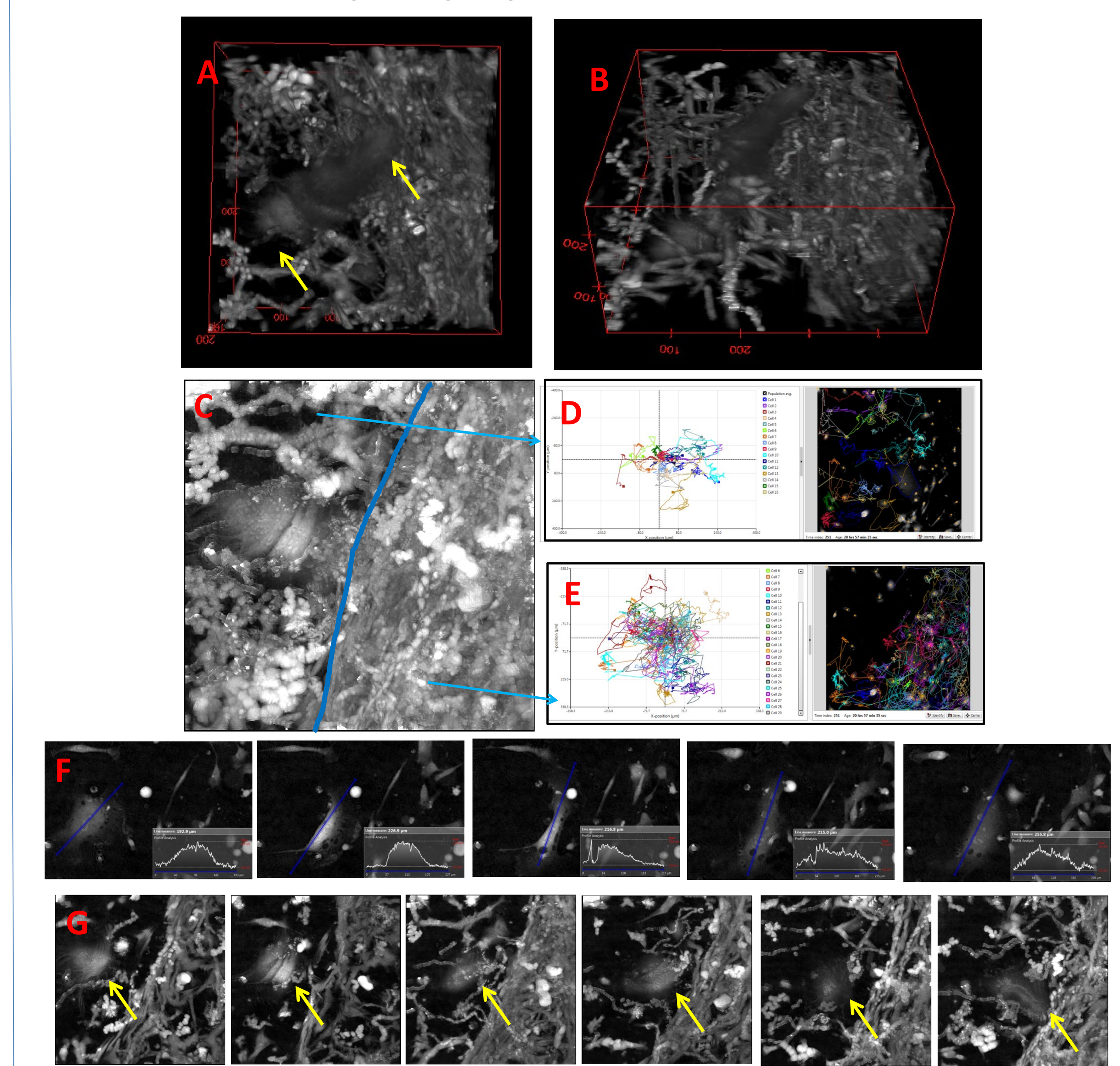
- The individual frames are color coded with increasing pixel density color coded from blue to green to yellow to red.
- The most notable features are the large number of red events at 48 hours in the PBS control, and the increasing number of smaller events and debris in the TAT/CLV treated samples, indicators of cell death.



- Mitotic cells have high optical thickness values, but not exclusively. Cellular debris tends to have low optical thickness values but again, not exclusively.
- As the complexity of the formulations increases, there is a decrease in the high optical thickness and a corresponding increase in the low optical thickness consistent with increasing cell death.

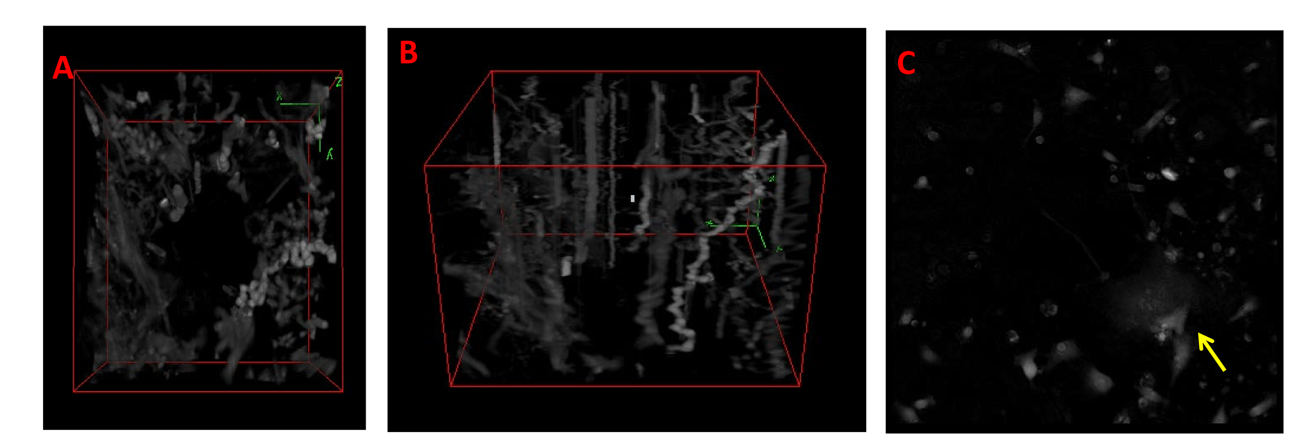
View of the tumor microenvironment (TME)

Day 2 analysis of TAT/CLV DOX miRNA 34A



- Top (**A**) and front (**B**) views of the 4D TME.
- Summed Z-stack image showing two distinct types of TME(**C**) formed on the plastic bottom of the Petri dish (left) and on the glass coverslip, at a 500 um lower elevation (right).
- Cell motility patterns of the plastic zone (**D**) and the glass zone (**E**).
- Two hour time course of a giant amoeboid phenotype HT-1080 cell (**F**).
- Twenty four hour tracking of the giant cells migration (**G**).
- .

Day 3 analysis of TAT/CLV Dox miRNA 34A



- Top (**A**) and front (**B**) views of the same 4D TME for hours 48 to 72.
- Location of the same giant cell at time point 48.

Summary:

- The HoloMonitor M4 enabled long-term live cellular analysis, tracking cells in a label-free manner with quantitative data linked to images and videos of any cell in the analysis at any time-point.
- By analyzing the HoloMonitor data in Image J we developed a novel 4-D holographic imaging method following XY positions of the cells and changes in the cellular thickness over time. We used this technique to characterize the population dynamics of untreated HeLa cells, and those treated with free Doxorubicin, revealing the morphology of dying cells in 4D plots.
- We introduced a simplified method for creating an extra-cellular matrix - instead of embedding cells within the matrix, we allow the cells to adhere to the substrate followed by matrix overlay.
- We applied the newly developed methodologies to an ongoing study, where the individual components of a complex nanoparticle formulation for Doxorubicin delivery were tested on HT-1080 cells.
- Our example of HT1080 cells treated with dox clearly shows the superiority of the 3D model, an important step in developing assays that better emulate multi-dimensional biological processes and offer the possibility of evaluating effects of drugs at lower cost and experimental complexity than those of in vivo assays.