Measuring apoptotic effects of EP4A1 and EP4A2 on Kuramochi with a high-throughput multiplex image cytometric method

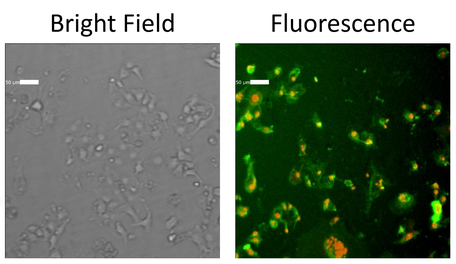
Jocelyn Reader[1,2], Sergio Mojica[3], Mackenzie Pierce[3], and Leo Li-Ying Chan[3]

[1]*University of Maryland Eastern Shore, School of Pharmacy and Health Professions, Department of Pharmaceutical Sciences, Princess Anne, MD 21853*
[2]*University of Maryland Greenebaum Comprehensive Cancer Center, Baltimore, MD 21201*
[3]*Department of Advanced Technology R&D, Revvity Health Sciences, Inc., Lawrence, MA 01843*

1. ABSTRACT

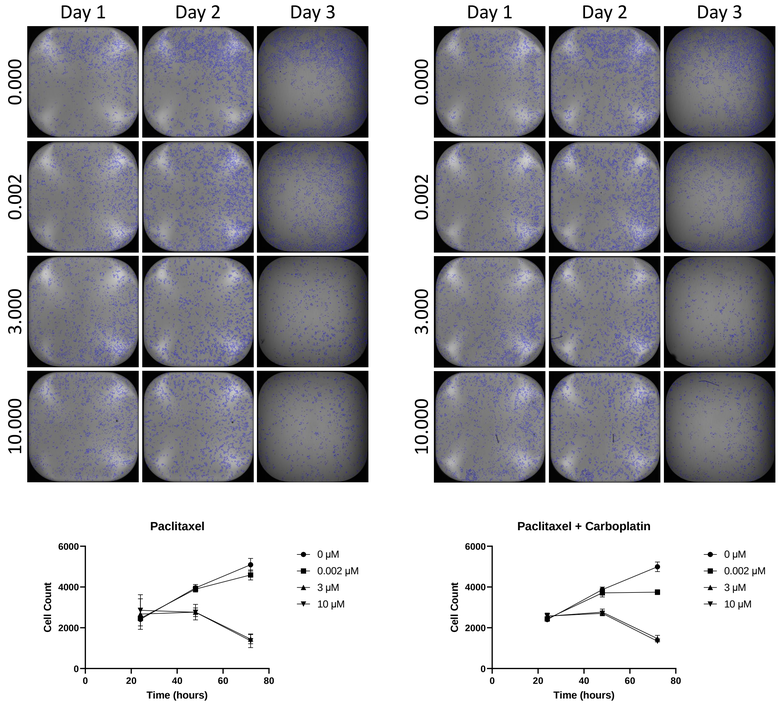
Ovarian cancer accounts for approximately 6% of all cancer death in women and has one of the highest mortality rates out of all gynecologic malignancies in the United States. Ovarian cancer can exhibit innate or acquired chemoresistance behavior, thus it is critical to identify novel therapeutic drug candidates. One of important characteristics of identifying potential chemotherapy drug candidate is the ability to induce apoptosis or programmed cell death in the target cancer cells. In order to measure apoptosis, various biomarkers are commonly employed such as Caspase 3, 7, 8 or 9, as well as Annexin V, which are all part of the cascade in apoptotic intrinsic pathway. However, typical fluorescent staining for image cytometry is not multiplexed that required multiple steps in order to determine the different type of apoptotic populations. In this work, we demonstrate a multiplexing apoptosis detection method to investigate the apoptotic effects of EP4A1, EP4A2, Paclitaxel, and Carboplatin on Kuramochi ovarian cancer cells. We employed the use of a high-throughput plate-based image cytometer (Celigo®, Revvity Health Sciences Inc.) to image and analyze drug-treated Kuramochi cells stained with Annexin V-APC, Caspase 3/7 (488), and propidium iodide (PI) to assess the early- and late-stage apoptosis, as well as necrosis. We expected to see an increase in dual staining with the addition of EP4A to paclitaxel and carboplatin; however, the more interesting data was observed by separating single positive cell populations (Caspase, Annexin or PI only) from dual positive cell populations (Annexin + PI, Caspase + PI, Annexin +PI). The results show that treatment with EP4A lead to a time dependent increase in Caspase 3 cleavage but only in the single positive cell population. This trend was also observed in Annexin V single positive cell populations which is an indication of early apoptosis. The additive effect observed with the addition of EP4A was not seen in the dual positive cell populations. The high throughput nature of the image cytometry also allowed us to easily and quickly determine the optimal treatment conditions for this experiment. We were able to record multiple time points and not only capture proliferation, but also simultaneously collect data on caspase cleavage, Annexin-V staining and PI staining on multiple concentrations of compounds in monotherapy, dual therapy, and triple therapy combinations. Typically, it may be difficult to test multiple conditions in duplicate and triplicate using flow cytometry, while Celigo® Image Cytometry may be used to obtain more robust data, and simultaneously acquire data of proliferation and multiplex staining with Annexin-V, PI and Caspase 3/7.

2. CELIGO® IMAGE CYTOMETRY



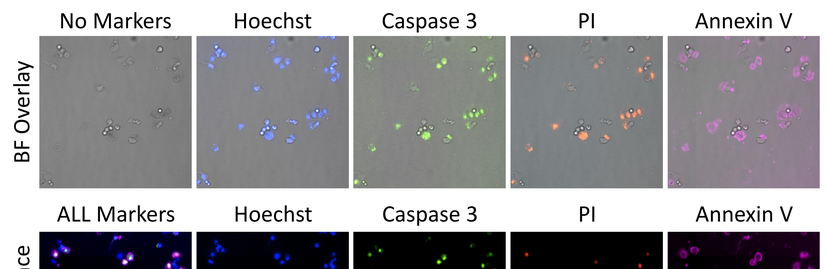
1. Celigo® is a plate-based image cytometer that can acquire bright-field and fluorescent images of entire well of standard microplates
2. The captured images are analyzed with the Celigo software to measure cell count, confluence, size, morphology, and fluorescent intensity
3. General Applications: Cell proliferation kinetic data, GFP/RFP expression, tumor spheroid size change, DNA cell cycle analysis, apoptosis, ADCC cytotoxicity, cell permeability and transwell assays.

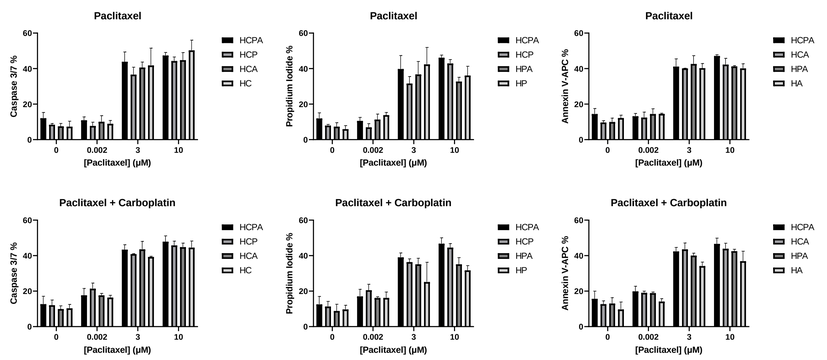
3. DRUG EFFECTS ON CELL PROLIFERATION



- Kuramochi ovarian cancer cells were treated with paclitaxel and a combination of paclitaxel with carboplatin for 72 h
- Label-free bright field images were acquired up to 72 h and directly counted using the Celigo image analysis software
- Direct cell counting results showed that both paclitaxel and the combination treatment induced dose dependent cytotoxicity effects
- Interestingly, the combination treatment of paclitaxel and carboplatin showed slightly higher effect for the 0.002 µM treatment
- On the final day, the cells were stained with Hoechst, Caspase-3, propidium iodide (PI), and Annexin V-APC for apoptosis and necrosis analysis

4. VERIFICATION OF APOPTOSIS MULTIPLEXING





- To verify apoptosis multiplex staining, Kuramochi cells were stained with combinations of Hoechst (H), Caspase-3 (C), PI (P), and Annexin V-APC (A)
- We measured the % of Caspase-3, PI, and Annexin V and compared the different combination of staining to ensure they were consistent
- The results showed that the full panel staining was comparable to two-color and three-color panels

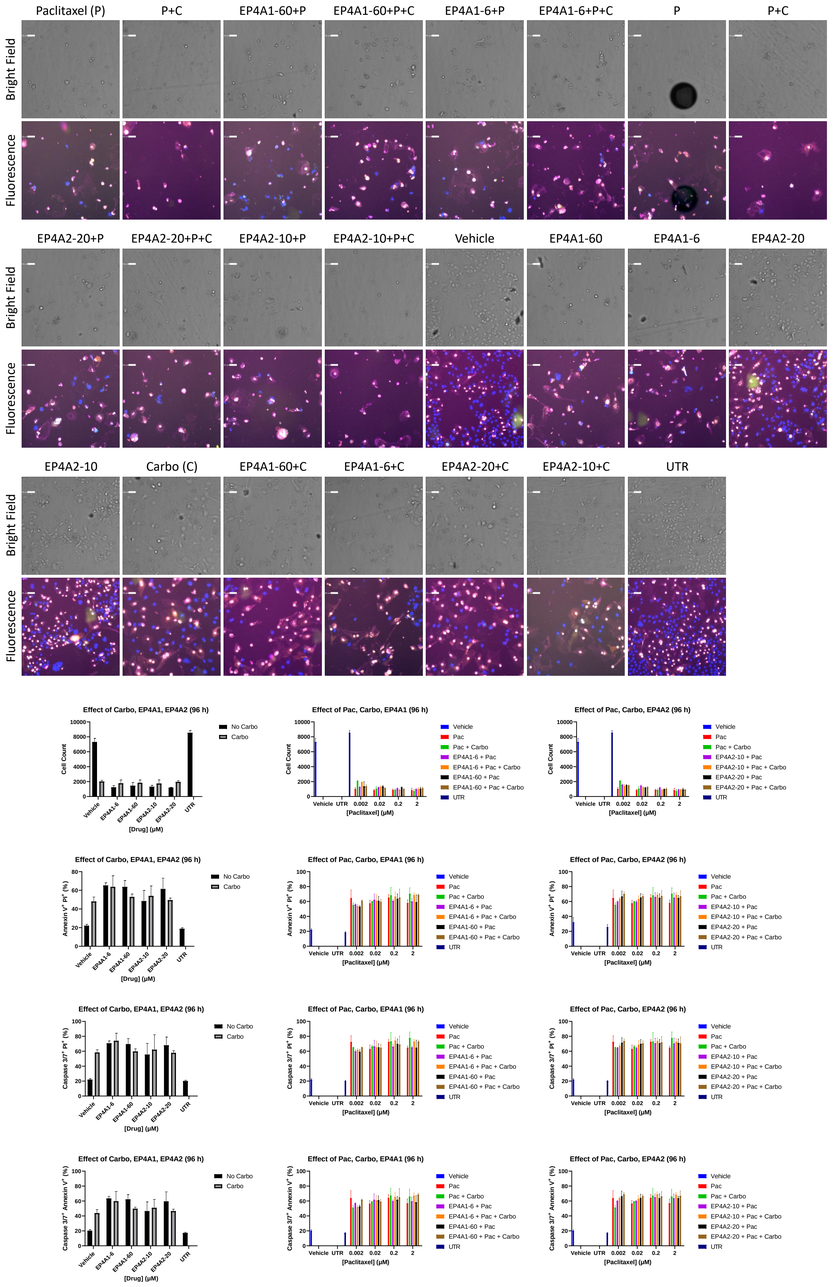
5. HIGH-THROUGHPUT APOPTOSIS SCREENING

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D			13	14	15	16	17	18	19	20	21	22	23											
E																								
F		Pac (µM)																						
G		2																						
H		0.2			1			2			3			4			5			6				
I		0.02																						
J		0.002																						
K		2																						
L		0.2			7			8			9			10			11			12				
M		0.02																						
N		0.002																						
O																								
P																								

- Kuramochi cancer cells were seeded in the three 384-well plates for drug treatment and time-course measurement at 48, 72, and 96 h

1. Pac
2. Pac + Carbo
3. EP4A1-60 + Pac
4. EP4A1-60 + Pac + Carbo
5. EP4A1-6 + Pac
6. EP4A1-6 + Pac + Carbo
7. Pac
8. Pac + Carbo
9. EP4A2-20 + Pac
10. EP4A2-20 + Pac + Carbo
11. EP4A2-10 + Pac
12. EP4A2-10 + Pac + Carbo
13. Vehicle
14. EP4A1-60
15. EP4A1-6
16. EP4A2-20
17. EP4A2-10
18. Carbo
19. EP4A1-60 + Carbo
20. EP4A1-6 + Carbo
21. Vehicle
22. EP4A2-20
23. EP4A2-10

6. EFFECTS OF EP4A1 AND EP4A2 ON APOPTOSIS



- Cell counts and percentages of double positive Caspase-3, PI, and Annexin V were measured from the Celigo® image cytometric analysis
- Additional analysis can be performed for single markers of Caspase-3, PI, and Annexin V

7. CONCLUSION

- Celigo® Image Cytometer enabled high-throughput screening of drug treatment for monitoring apoptosis and necrosis effects
- By multiplexing Caspase-3, PI, and Annexin V-APC, researchers may directly obtain population percentages without the need to set up 2 separate assays
- The ability to perform high-throughput apoptosis/necrosis analysis allows more parameters to be captured for improved drug characterization