Manuscript No: JoVE60185
Manuscript title: Generation of heterogeneous drug stress gradients across cancer populations on the microfluidic Evolution Accelerator for real-time observation
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Reviewer #2:

Major Concerns:
None.

Minor Concerns:
This paper introduces the Evolutionary Accelerator, a microfluidic cancer-on-chip model which reproduces important features of a complex tumor microenvironment, while enabling robust quantitative descriptions of cancer dynamics. Specifically, it allows cancer cells to inhabit an interconnected array of microenvironments while generating a heterogeneous concentration landscape (of chemotherapeutics and other chemical stress, or possibly nutrients). This allows long-term, real-time monitoring of cancer evolutionary dynamics.
This is an innovative and elegant system that is utterly needed considering the high cost and difficulties of mouse models in addition to their questionable relevance to human cancer progression on the one hand, and the insufficient complexity of 2D cultures on the other hand. The system and its capabilities are very well presented through images, the protocol and demonstrations (cell growth and motility assays). Therefore, I recommend publication, while suggesting that the Authors address the following comments (optional).
(1) Line 293: doubling time in conventional cultureware is 24 hours. Can this be supported by data or a reference?
(2) A review paper that may be relevant and may be worth citing is, "Towards control of cellular decision-making networks in the epithelial-to-mesenchymal transition", Phys Biol. 16(3):031002 (2019).
(3) "Microfluidic cancer-on-chip models 38 have been prophesied to bridge the gap between the oversimplified conventional 2D cultures" -prophesied should probably be proposed.
(4) The system is an "evolution accelerator" - a few words on how it accelerates evolution, referring to earlier work (Death Galaxy) by the authors would be great.
(5) Are the microfluidic pattern template file and the plate holder pattern available?
(6) More information would be useful on point 3.2.5 in disinfecting with UV exposure- for how long?
(7) May be worth mentioning that not all 3 lumox dishes/chambers need to be used at the same time.
(8) It would be useful to clarify if any parts of the protocol are optional, and which are interchangeable. Are any variables interchangeable or modifiable? E.g., 3.4.10 flow rate, does it have to be 20uL/hr?
(9) Microscopes reliant on perfect focus, won't be able to focus on the interface of the PDMS/lumox chip automatically. The authors should cover this information in detail and suggest solutions. In general, they should comment on the portability of the system to a standard research lab.
(10) Are there any regions in the chip where cells did not grow well?

(11) Do the authors observe wash off of cells that escape out of the culture region? If so, how common is it and how much of the population can be lost in such a way?

Authors' response: Thank you very much for your kind words and comments. We really appreciate your feedback.

(1) A paper has been referred to support the presented doubling time:
[13] Lin, K. C. et al. The role of heterogeneous environment and docetaxel gradient in the emergence of polyploid, mesenchymal and resistant prostate cancer cells. *Clinical & experimental metastasis*. **36** (2), 97-108 (2019).

(2) The suggested review article is now cited in the Discussion section:
"With the ability to control and monitor the behaviors of mixed tumor cell populations under well-controlled stress gradients, our microfluidic EA technology may also serve as a platform to study regulatory mechanisms and the phenotypic transformation involved in EMT in the context of cancer therapeutic strategies.[22]"

(3) The sentence now reads: Microfluidic cancer-on-chip models have been proposed to bridge the gap …

(4) A few sentences have been added in the second paragraph of the Introduction section:
"The design of the Evolution Accelerator is based on our previous work, in which the evolutionary dynamics of organisms in a metapopulation can be accelerated.[10-11] Specifically, in a group of spatially separated populations which interact at some level, when exposed to a heterogeneous stress landscape, the most fit species can dominate in a local population faster compared with that of a large uniform population. The advantageous species then migrate to neighboring microhabitats in search of resources and space, and eventually dominate the entire population."

(5) The EA pattern template files ("EA_large-gap.gds", "EA_small-gap.gds") and the 3D CAD file ("3-well plate.FCStd") are now included in the supplementary materials and on GitHub.

(6) The description in the protocol has been modified:
"3.2.5. Disinfect the PDMS devices via UV exposure for at least 1 hour and leave in a sterile environment."

(7) A sentence has been added in the protocol:
"3.4.2. … Note that the 3 wells are independent, and 3 experiments can be done separately."

(8) The variables and procedures in the protocols have been optimized. We suggest the readers to follow our recipe if there's no special requirement. For instance, if the flow rate is set to be higher than 100 µL/hr, the cells near the peripheral habitats would be affected by the advection flow and do not grow well. If the flow rate is set to be lower than 1 µL/hr, the flow is not sufficient to maintain fixed bio-chemical boundary condition in the media channels.

(9) More description is now added in the protocol:

4.2. Configure software to acquire images across two channels at 10X magnification for each chip with automatic image-stitching after one round of autofocus. Be aware that Perfect Focus does not guarantee satisfying image quality. It is more recommended to generate a customized focus surface for long-term image acquisition based on either autofocus or manual focus across the chip.
4.3. Take images every hour and leave experiment running on the time scale of weeks. Monitor image quality on a daily basis and update the focus surface if necessary.

(10) If strictly following the suggested protocols, there should be no obvious variation in terms of cell proliferation rate across the chip.

(11) The width of the slits (15 μm) is design to prevent cells from escaping the culture region, but this can definitely happen. The portion of the cells that "extravasated" into the media channels depends heavily on cell types. Typically, the probability of each cell getting into the media channels ranges from 0.005 to 0.02 (1/hour). Mesenchymal cells have higher escaping rate then epithelial phenotypes.