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PRINCIPAL INVESTIGATOR: Robert Clarke, PhD, DSc

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Robert Clarke & Yue Wang

E-Mail: clarker@georgetown.edu & yuewang@vt.edu

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
More women die from the estrogen receptor positive (ER+) breast cancer subtype than from any other. The proportion of early ER+ recurrences (=5 years since diagnosis) approaches that for all triple-negative breast cancers alone. Late recurrences (>5 years after diagnosis), the result of dormancy, are most common in ER+ disease and can arise decades after the initial diagnosis. Since recurrent breast cancers have escaped the effects of endocrine therapies, and are lethal, we will study endocrine resistance (Tamoxifen; Fulvestrant). Our primary objective is to identify what drives breast cancer growth and determine how to stop it. We will learn about why some breast cancers are aggressive and others are indolent, and why/how some breast cancers lay dormant for years and then re-emerge.

15. SUBJECT TERMS
Breast cancer, drug resistance, admixing, ecology, multiscale modeling

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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1. Introduction

~70% of newly diagnosed breast cancers are ER+ [1]. Many of these women die because metastatic ER+ disease becomes treatment resistant. Resistance is multiscale, i.e., evident at many levels, with genetic, cellular, and phenotypic features (including intratumor heterogeneity; ITH), all are molecularly manifested, and functionally realized, as networked changes in the transcriptome and proteome. We will take a systems biology approach to portray the proteome and transcriptome topology of treatment-induced adaptive remodeling of cell admixtures in vitro and in vivo. Overarching goals are to understand the principles of this remodeling and uncover the mechanisms that confer endocrine resistance in breast cancer, leading to new treatment strategies.

NB: Please note that because of Covid-19 restrictions at Georgetown and at Virginia Tech, we have lost almost 6 months of time. Please also note that Dr. Clarke is moving to the University of Minnesota at the beginning of October, 2020. To enable the work to continue as planned, a request to transfer the remaining funds for this award will be forthcoming shortly. It is likely that the move from DC to MN will cause additional delay in some (but not all) of the work proposed. This will be clarified in the next annual report.

2. Keywords

Drug resistance, admixing, ecology, multiscale modeling

3. Accomplishments

A) Major goals (and related subtasks) of the project from approved SOW:

SPECIFIC AIM 1 (specified in proposal)	Timeline	Site 1	Site 2	Percent complete	Date completed (if 100%)
Major Task 1 (Aim 1a)	Months				
Subtask 1: Determine prevalence of **R** and/or **P** cells in **S** populations	1-6	Dr. Clarke Dr. Sengupta			
Subtask 2: Determine the effects of different **S**:**R** ratios on response to TAM and ICI in MCF-7, LCC, T47D, and ZR-75-1 **S** and **R** matched cell models *in vitro*	1-18	Dr. Clarke Dr. Sengupta			
Milestone(s) Achieved: Identified effects of **S**:**R** ratio on responsiveness to TAM and ICI *in vitro* in multiple breast cancer cell models and identified optimal admix ratios for *in vivo* studies					
Local IACUC approval (annual renewal required only – approval for studies already in place)	1	Dr. Clarke		100%	8/31/19
Local IRB approval (add this award as an exemption for use of existing data – no new clinical data will be generated in this BT#2)	1	Dr. Clarke		Pending	
Subtask 3: Determine the effects of different **S**:**R** ratios on response to TAM and ICI in **S**:**R** matched cell models *in vivo* (models and admixes guided by the optimal* results in Major Task 1/Subtask 2) It is difficult to provide direct numbers until the *in vitro* work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone ± ICI (15+15=30) S cells alone ± ICI (15+15=30) R+S cells at a single ration ± ICI (15+15=30) Total = 90/experiment We may do 3 such experiments over the 18-month period for 270 mice.	6-24	Dr. Clarke Dr. Sengupta		10%	
Milestone(s) Achieved: Identified effects of **S**:**R** ratio on responsiveness to TAM and ICI *in vivo*					
Major Task 2 (Aim 1b)					
Subtask 1: Determine the role of GJIC in the ability of **R** to make **S** cells resistant to TAM and ICI in MCF-7, LCC, T47D and ZR-75-1 matched cell models *in vitro* (guided by the optimal experimental conditions from Aim 1a)	6-18	Dr. Clarke Dr. Sengupta		20%	
Subtask 2: Determine the role of microvesicles and protein secretion	6-18	Dr. Clarke Dr. Sengupta			

(transwell) in the ability of R to make S cells resistant to TAM and ICI in MCF-7, LCC, T47D and ZR-75-1 matched cell models *in vitro* (informed by the optimal experimental conditions identified in Aim 1a)					
Milestone(s) Achieved: Identified role GJIC, microvesicles and protein secretion (transwell) in the ability of **R** to make **S** cells resistant to drug and how this is affected by different **S**:**R** ratios. Identified conditions to allow design and execution of *in vivo* studies with guggulsterone and/or GW4869 (experiments will be done if supported by data and if time permits)					
Subtask 3: Collect and store materials (*e.g.,* cell lysates) from optimal conditions for omics studies in Aim 2	1-24	Dr. Clarke Dr. Sengupta			
SPECIFIC AIM 2 (specified in proposal)	**Timeline**	**Site 1**	**Site 2**		
Major Task 3 (Aim 2a)					
Subtask 1: Collect RNA and protein from the materials stored from Aim 1a (this will be done as the optimal experiments are identified above)	1-24	Dr. Clarke Dr. Sengupta	Dr. Wang	50% Site 2	
Subtask 2: Perform array and proteome data collection, processing of raw data from Major Task 3/Subtask 1 (above), and *initial* analyses (*e.g.,* CAM, kDDN)	1-24	Dr. Clarke Dr. Sengupta	Dr. Wang	50% Site 2	
Milestone(s) Achieved: Create initial signaling maps of what is communicated by **R** to **S** to confer resistance and how this is affected by different **S**:**R** ratios					
Major Task 4 (Aim 2b)					
Subtask 1: Build initial mathematical models of cell population remodeling dynamics (*in vitro* and *in vivo* data)	4-24	Dr. Bansal		50%	
Subtask 2: Build final mathematical models of cell population remodeling dynamics (*in vitro* and *in vivo* data)	24-36	Dr. Bansal			
Milestone(s) Achieved: Identified how endocrine therapies and the starting ratios of **S**:**R** cells affects population responses to treatment					
Major Task 5 (Aim 2c)					
Subtask 1: Use the data from Aims 1 and 2 to design and execute novel drug combination and scheduling studies *in silico* (mathematical modeling), *e.g.,* ICI+DNMTi	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Subtask 2: Design and execute novel drug combination and scheduling studies *in vitro* using the predictions in Major Task 5/Subtask 1	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified novel optimized (activity vs. toxicity) combination regimens *in vitro*.					
Subtask 3: A small number of predictions from the *in vitro* modeling in Major Task 5/Subtask 2 will be	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	

tested *in vivo* (we anticipate completing ~5 such animal studies) It is difficult to provide direct numbers until the *in vitro* work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone + Vehicle (15) S cells alone + Vehicle (15) R cells alone + Drug A and + Drug B (15+15=30) S cells alone + Drug A and + Drug B (15+15=30) R+S cells at a single ratio with Vehicle, + Drug A and + Drug B (15+15+15=45) Total = 135/experiment We may do 4 such experiments over the funding period (n=540 maximum number mice).					
Milestone(s) Achieved: Identified novel optimized (activity vs. toxicity) combination regimens *in vivo*.					
SPECIFIC AIM 3 (specified in proposal)	**Timeline**	**Site 1**	**Site 2**		
Major Task 6 (Aim 3a)					
Subtask 1: Initial CAM and kDDN modeling of microarray data from human tumors (public and in-house datasets); data will be fed back to Aim 2 to increase clinical relevance	1-12		Dr. Wang	100% Site 2	8/31/19
Subtask 2: Update models using outcomes from Aim 2 and study if candidate molecules from Aim 2 are associated with clinical outcome (univariate and multivariate)	12-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified clinically relevant molecules associated with ITH and endocrine resistance					
Subtask 4: A small number of predictions from the *in vitro* modeling in Major Task 5/Subtask 3 will be tested *in vivo* (~5 such experiments will be done) A small number of predictions from the in vitro modeling in Major Task 5/Subtask 2 will be tested in vivo (we anticipate completing ~5 such animal studies) It is difficult to provide direct numbers until the in vitro work is completed. A standard design for a single would include the following (n=15/group as in application): R cells alone ± Drug A (15+15=30) S cells alone ± Drug A (15+15=30)	18-36	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	

R cells alone ± Drug B (15+15=30) S cells alone ± Drug B (15+15=30) R+S cells at a single ratio + Drug A + Drug B (15+15=30) Total = 150/experiment We may do 3-5 such experiments over the funding period (n=750 maximum number mice).					
Milestone(s) Achieved: Identified novel therapeutic strategies for ER+ breast cancer to prevent, delay or reverse resistance, and do so within minimized toxicity. These insights could be used to design clinical trials to be done outside this research program.					
Major Task 7 (Aim 3b)					
Subtask 1: Test candidate molecules from the model predictions in Aims 2 and 3a. For example, as described in the narrative section, genes upregulated in resistant cells relative to sensitive cells will be overexpressed (cDNA; regulable and/or constitutive promoters) in sensitive cells and knocked down in resistant (RNAi) if their mRNA or protein is still present in sensitive cells. The gene will be knocked out (CRISPR) in resistant cells if the gene is known to be lost or expression is undetectable in sensitive cells. The reverse experiments will be done where a gene is down regulated or lost in resistant cells relative to its expression/presence in sensitive cells.	12-36 months	Dr. Clarke Dr. Sengupta Dr. Bansal	Dr. Wang	10% Site 2	
Milestone(s) Achieved: Identified mechanistically relevant molecules associated with ITH and endocrine resistance					

B) What was accomplished under these goals?

Aim 1. Summary

To determine the effects of co-culture of sensitive and resistant cells (S:R; 1:1 ratio) in response to Fulvestrant (ICI) in estrogen receptor positive (ER+) breast cancer cells, we used fluorescently labelled sensitive (S) and resistant (R) variants for MCF7-derived LCC1 (S) and LCC9 (R) cells. The cells were grown in either mono-culture or co-culture in the ratio of 1:1. Cells were treated with vehicle (0.1 % ethanol) or Fulvestrant (500nM) for 4 days. A 12-day Fulvestrant treatment was conducted but we were unable to perform flow cytometry due to closure of core facilities at Georgetown-Lombardi Cancer Center due to COVID-19 pandemic. As detailed in our prior report, we used Cyto-phase violet dye (Biolegend) that is used for live cells to determine the cell cycle phases. The cells were gated for their fluorescent labels (eGFP for LCC1 and mCherry for LCC9) before assessing for cell cycle phases.

A four-day treatment with 500nM Fulvestrant showed a marked decrease in 'S' phase cells of LCC1 but not in LCC9 cells under monoculture conditions (Figure 1). In co-culture conditions, where LCC1 and LCC9 cells were cultured in 1:1 ratio, Fulvestrant treatment marginally lowered Fulvestrant-induced reduction of 'S' phase cells of sensitive LCC1 cells compared to vehicle treatment (p=0.08; Figure 2, left panel). No major changes were noted in G1 and G2/M cell phases. The LCC9 cells in the co-culture showed reduction of G1 cells (p=0.03) with Fulvestrant when compared with mono-culture under identical treatment (Figure 2, right panel).

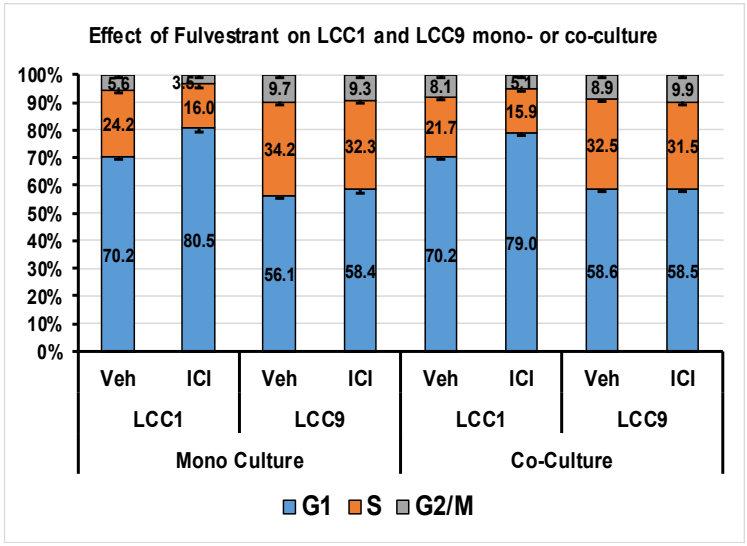
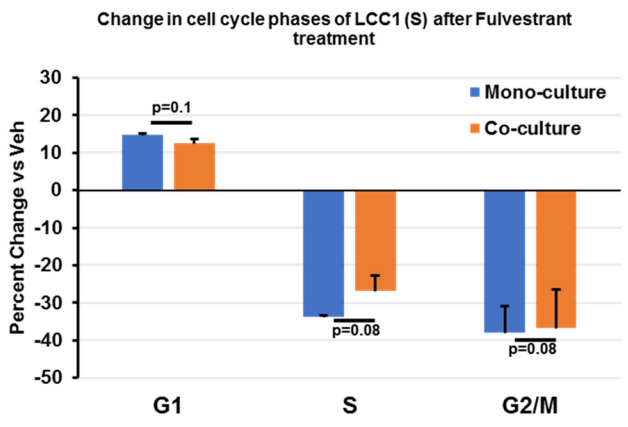


Fig 1. Effect of co-culture on Fulvestrant sensitivity in LCC1 (sensitive) and LCC9 (resistant) cells.



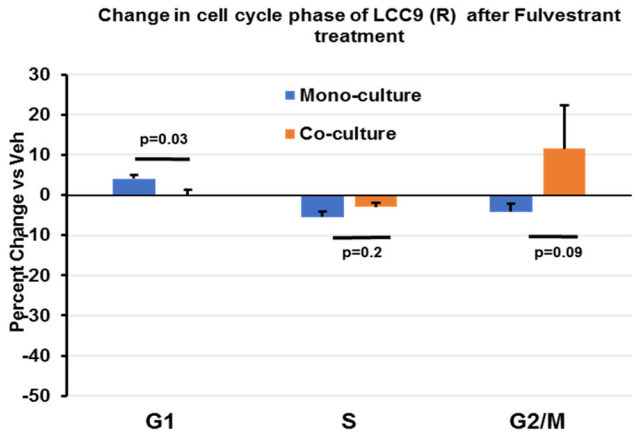


Fig 2. Effect of co-culture on Fulvestrant sensitivity in LCC1 (sensitive) and LCC9 (R) cells. The p values (t-test, two-tailed, paired) are mentioned for each cell phases comparing between mono- and co- culture conditions.

Aim 2B Summary:
Aim 2B of our project focuses on the ecology of cell population dynamics. From an ecological view, populations of susceptible **(S)** and resistant **(R)** cells are populations of different "species", while GLC, GLN, and O_2, are "resources" necessary for the two populations to grow. Our focus here is to study how resistant (R) and sensitive (S) cells interact to alter the response of S+R populations to treatment.

Aim 2B Progress:
We have made significant progress on this aim so far:

- We have generated *in vitro* population dynamics data from mono- and co-cultures of fluorescence-tagged LCC1 and LCC9 cells.
- We have developed a new image processing algorithm for automated cell counting.
    - Statistical denoising allows for more accurate counts and limits measurement error, which had impeded off-the-shelf algorithms (e.g. ImageJ).
    - The algorithm can process thousands of images in minutes, allowing for fine-scale measurement of growth across time.
        - Allows for data-intensive ("Big Data") inference approach.
    - This algorithm is implemented in Python and is open-source.
    - We have validated our results of the automated counts with manual counts done in the lab, and our results are very positive. (See Figure 1, Figure 2).
- We have developed two mathematical models to capture the population dynamics of single and interacting populations based on the Generalized Lotka-Volterra interaction model.
    - These models are able to partition uncertainty across the parameter space, enabling precise inference of growth behavior while still accounting for variation due to the measurement process.
- We have developed a statistical Bayesian inference framework for this population dynamics mathematical model to infer the growth and interaction parameters from the *in vitro* data of the two populations.
    - This framework is based on a hierarchical Bayesian model implemented in Python using the Stan platform.
    - This approach is able to represent uncertainty both at each time point and across the entire time series due to the generative modeling approach.
- Using the Bayesian inference framework, we have identified intrinsic growth and capacity parameters from the mono-culture data and the impact of treatment on these parameters by fitting the population dynamics models. (See Figure 3a, 3b).
    - Precise identification of these parameters supports inference of ecological effects of populations grown in coculture
- Using the Bayesian inference framework, we have fit the coculture growth population time series with paired systems of equations, both with and without drug (See Figure 4a, 4b).
- We have identified a marginally amensal ecological relationship between cell populations from the co-culture data that becomes strongly competitive in the presence of drug. (See Figure 5).

Aim 2B Next Steps:

- Generate *in vitro* population dynamics data from additional replicates to decrease the uncertainty in inferred parameters.
- Confirm the inferred carrying capacity parameters with additional *in vitro* data.
- Use the Bayesian inference framework to suggest mechanisms for differences in response to treatment.
- Examine the robustness of the ecological relationships across a range of initial growth conditions (e.g. initial cell seeding ratio)

Products:

- [Presentation] Susswein, Z., Sengupta, S., Clarke, R., Bansal, S. “Borrowing ecological theory to infer interactions between sensitive and resistant cancer cell populations”. Chapman Seminar, Department of Biology, Georgetown University.

Figure 1: Result of the cell-counting algorithm we have developed. The red outlines show cells identified by the algorithm.

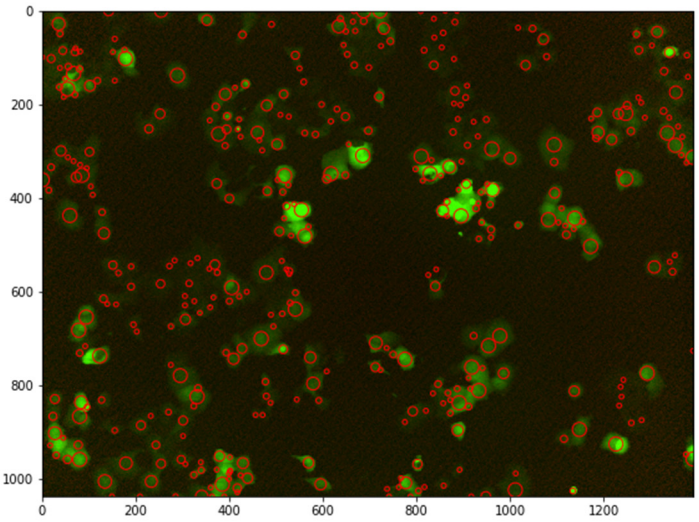


Figure 2: Validation of the cell-counting algorithm against human manual counts.

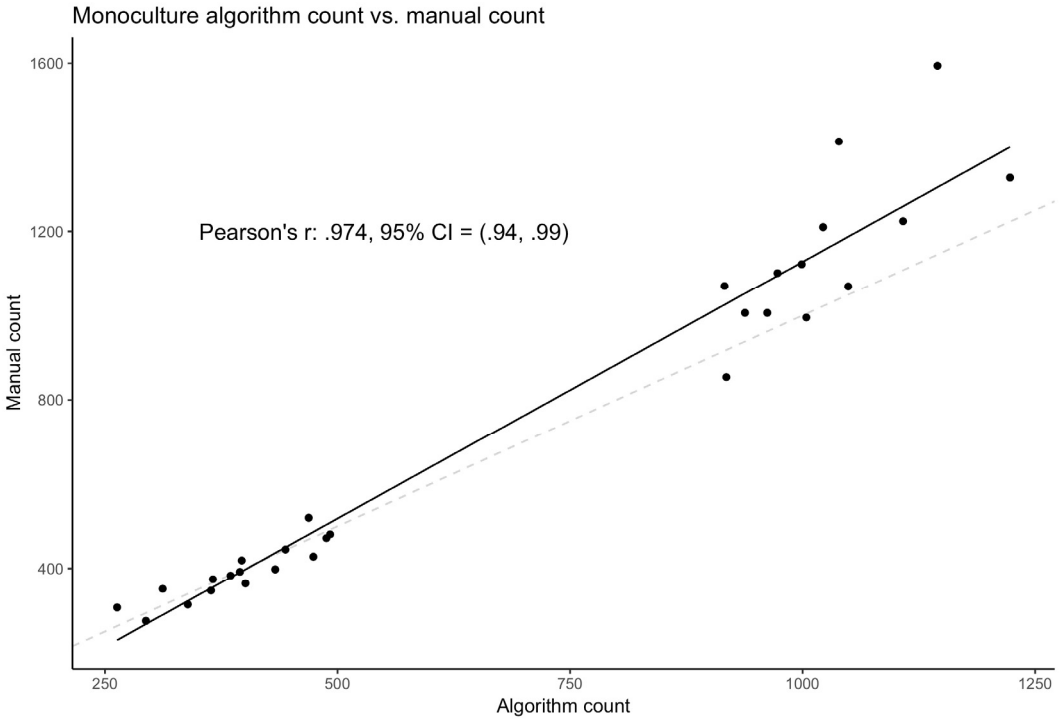
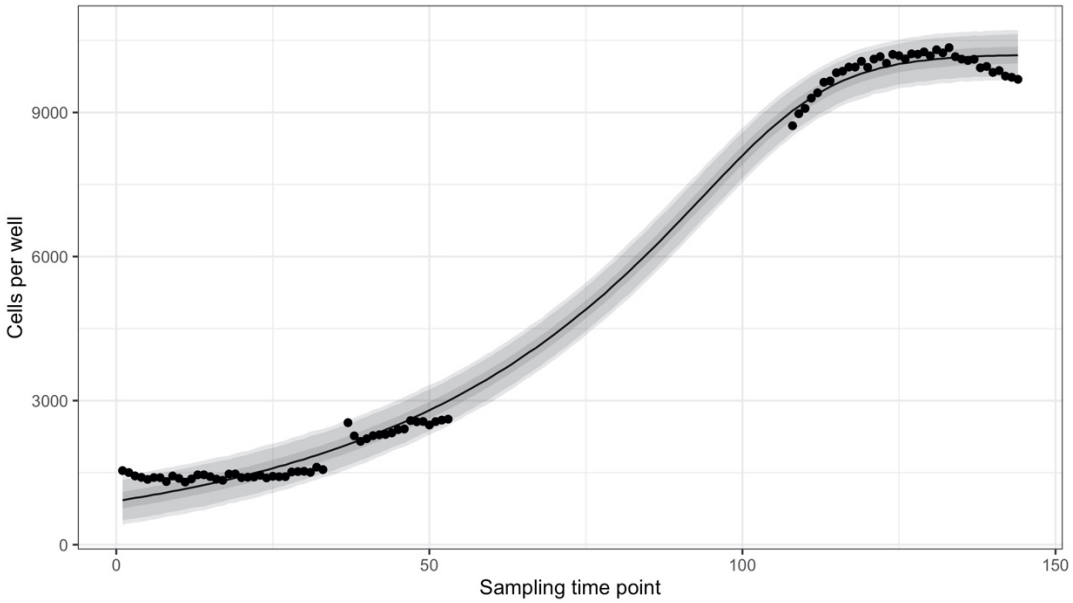


Figure 3a & 3b: Population dynamics of sensitive (LCC1) cell line without drug (a) and with drug (b). Note the difference in y axis scale. The points are the observed population counts and the black line is the inferred mean population growth trend from the Bayesian inference framework. The shaded regions represent 50%, 75%, and 95% Credible Intervals across time.

(a)



(b)

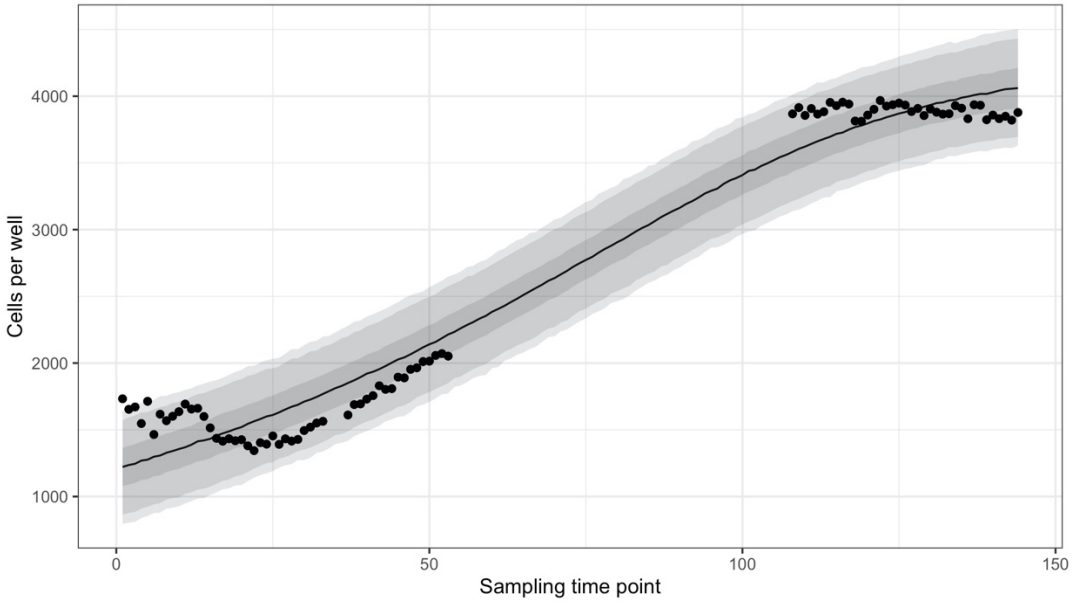
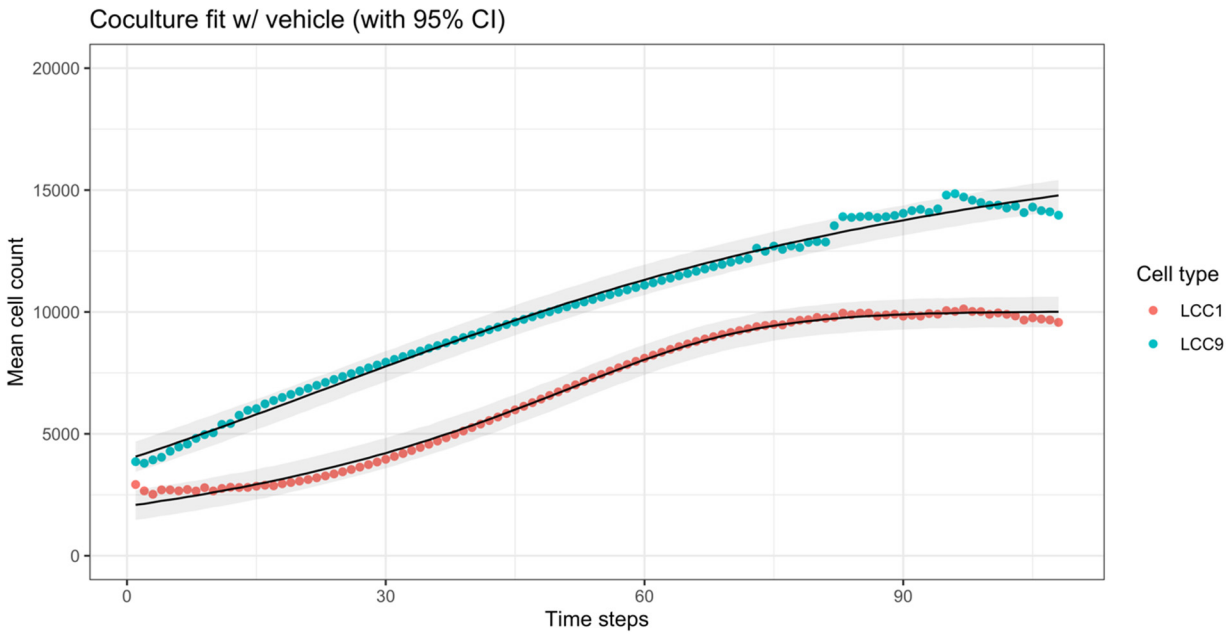


Figure 4a & b: Population dynamics of interacting sensitive and resistant cells without (a) and with (b) drug. The points are the observed population counts and the black line is the inferred mean population growth trend from the Bayesian inference framework. The shaded regions represent 95% Credible Intervals across time.

(a)



(b)

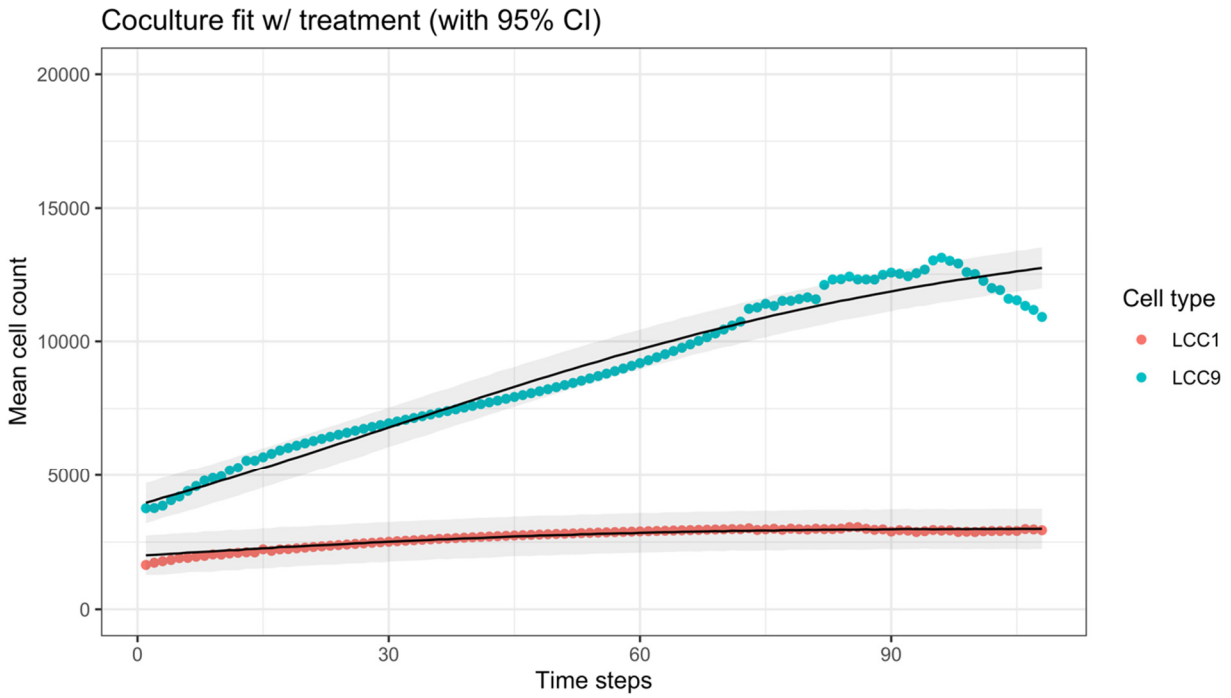
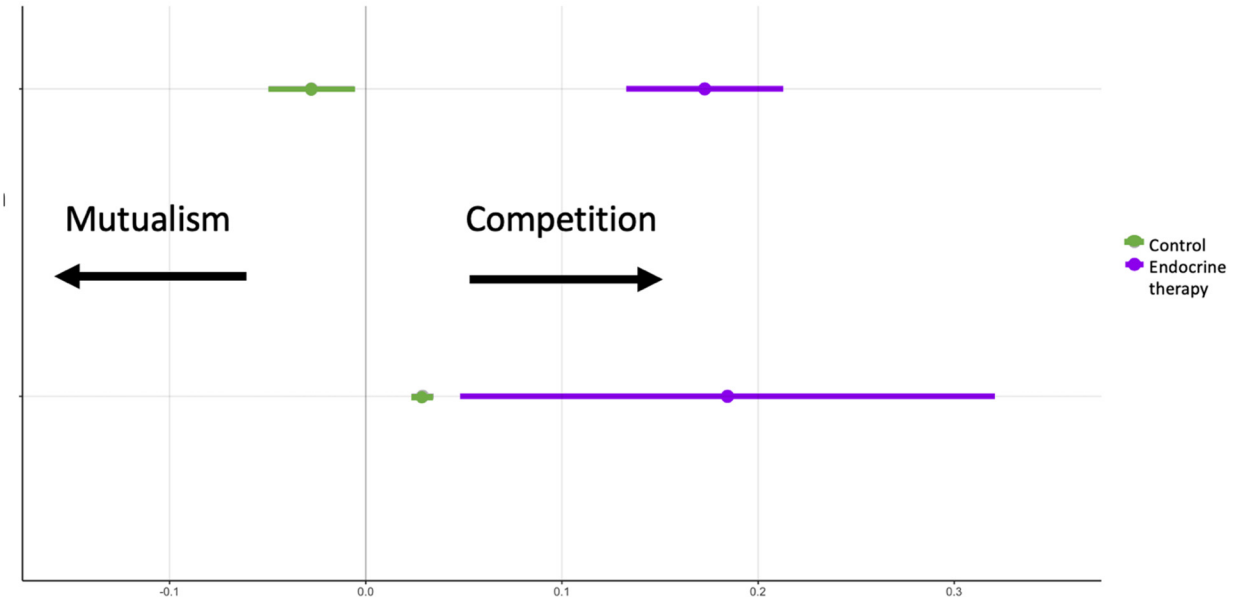


Figure 5: Cell populations have weak net ecological relationships in the presence of vehicle (green), but these become strongly competitive in the presence of treatment (purple). The two lines are the proportional impact of each population on the other population on the differential growth equation scale (log effect). Larger values indicate a greater competitive impact inhibiting growth in the other population.



C) What opportunities for training and professional development has the project provided?
Nothing to report (this project was not intended to provide training and professional development opportunities).

D) How were the results disseminated to communities of interest?
Nothing to report.

E) What do you plan to do during the next reporting period to accomplish the goals?

Aim 2B Next Steps:

- Generate *in vitro* population dynamics data from additional replicates to decrease the uncertainty in inferred parameters.
- Confirm the inferred carrying capacity parameters with additional *in vitro* data.
- Use the Bayesian inference framework to identify interaction parameters from the co-culture data with drug.

4. Impact

Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

Nothing to report.

5. Changes/Problems

Nothing to report.

6. Products

List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

Publications, conference papers, and presentations

Report only the major publication(s) resulting from the work under this award.

Journal publications.

Clarke, R., Tyson, J.J., Tan, M., Baumann, W., Xuan, J & Wang, Y. "Systems biology: quantitative multiscale modeling in research in breast and other cancers." *Endocr Relat Cancer,* in press, doi: 10.1530/ERC-18-0309, 2019.

Chen, L., Wang, N., Herrington, D.M., Clarke, R., Wu, C.-T. & Wang, Y. "debCAM: a Bioconductor R package for fully unsupervised deconvolution of complex tissues." *Bioinformatics,* 36: 3927–3929, 2020.

Fan, M., Xia, P., Clarke, R., Wang, Y. & Li, L. "Radiogenomic signatures reveal multiscale intratumour heterogeneity associated with biological functions and survival in breast cancer." *Nature Commun,* in press, 2020.

Clarke, R., Kraikivski, P., Jones, B.C., Sevigny, C.M., Sengupta, S. Wang., Y. A systems biology approach to discovering pathway signaling dysregulation in metastasis." *Cancer Metastasis Rev*, doi: 10.1007/s10555-020-09921-7, 2020.

Books or other non-periodical, one-time publications.

Other publications, conference papers, and presentations.

Website(s) or other Internet site(s)

Technologies or techniques

Inventions, patent applications, and/or licenses

Other Products

7. Participants & Other Collaborating Organizations

Name: Robert Clarke
Project Role: Principal Investigator
Research Identifier (ORCID ID): 0000-0002-9802-8241
Nearest person months worked: 2
Contribution to project: Dr. Clarke has served as Initiating PI, performing tasks as listed in SOW

Name: Surojeet Sengupta
Project Role: Co-Investigator
Nearest person months worked: 4
Contribution to project: Dr. Clarke has served as Co-Investigator, performing tasks at Georgetown University site as detailed in the project SOW

Name: Alan Zwart
Project Role: Technician

Nearest person months worked: 8
Contribution to project: Mr. Zwart assisted Drs. Clarke and Sengupta with laboratory and animal work at Georgetown during Year 01 of the project.

Name: Lu Jin
Project Role: Bioinformatician
Nearest person months worked: 2
Contribution to project: Mr. Jin works as project data analyst and Bioinformatician, and collects the laboratory data as it is generated to prepare it for work to be done by the mathematical modelers at Virginia Tech.

Name: Shweta Bansal
Project Role: Co-Investigator
Nearest person months worked: 1
Contribution to project: Dr. Bansal is a mathematical biologist who assists Dr. Clarke with experimental design and data analysis, primarily related to Specific Aims 2 and 3 as detailed in the SOW.

Has there been a change in the active other support of the PI or senior personnel since the last reporting period?
Nothing to report.

What other organizations were involved as partners?
Nothing to report (aside from Partnering PI institution, Virginia Tech University).

8. Special Reporting Requirements

The Partnering PI (Yue Wang, PhD) has submitted an independent annual report for this period.

9. Appendices