Agent-Based Simulations of *In Vitro* Multicellular Tumor Spheroid Growth

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Abstract

Multicellular tumor spheroids (MTS) are an *in vitro* model system of avascular tumors. They are used to study how local microenvironments affect cellular growth, viability, and therapeutic response. We are developing in silico, agent-based analogues to provide new options for gaining exploitable insight into both the *in vitro* and *in vivo* systems. MTS exhibit characteristic phenotypic attributes: initial exponential growth becomes linear and is followed by growth saturation and a stabilization of spheroid size; and a concentric layered structure, consisting of an outer shell of proliferating cells, an intermediate layer of viable but quiescent cells, and an inner necrotic core. Our agent-based, discrete event analogue consists of different spaces for tumor cells, oxygen, nutrient, and toxic inhibitors. It successfully simulates the three phases of spheroid growth and achieves the balance of cell gain and loss that is necessary for saturation. When exposed to varying levels of nutrient and oxygen, the model behavior is qualitatively similar to that observed *in vitro*. A goal is to have increasing overlap between the phenotype of the analogue in silico systems and the phenotype of their *in vitro* referents. The development process will necessarily expose gaps in the understanding of tumor spheroids and lead experimentalists in new and novel directions.

1. INTRODUCTION

Cancer is characterized by improper cellular proliferation. Cancerous cells do not undergo senescence, nor do they listen to regular signals to become apoptotic. In many forms of cancer, this cellular proliferation self-organizes into the growth of solid tumors. Cancer, and in particular the process of tumor growth, is difficult to study *in vivo*. Hence, much cancer research has focused on developing model systems [1], both *in vivo* animal models and *in vitro* cell culture models. Multicellular tumor spheroids (MTS) are an *in vitro* model system of early stage tumor growth that has been well developed and extensively studied. Here we report on the development and results of an initial agent-based model of MTS growth.

What is the motivation for developing such a model? Just as *in vitro* models provide insight into the *in vivo* system, we believe that reliable and robust in silico model systems can provide insight into *in vitro* systems and by extension into *in vivo* systems and eventually patients. Our in silico tumor cell agents incorporate aspects of tumor cell biology such as cellular metabolism, the cell cycle, and cellular responses to the effects of changing microenvironmental conditions. One issue that remains unsolved in tumor spheroid research is the action of the necrotic extract. It is unclear whether the necrotic extract slows the progression of cells through the cell cycle or causes more cells to arrest and become quiescent (or both). It is also unclear whether the cells arrest primarily due to exposure to the necrotic extract or if there are other factors, like cellular forces or contact inhibition that cause arrest. We aim to use our model to develop and explore hypotheses that have not been extensively studied or are difficult or impossible to study *in vitro*, and thereby generate new insights and avenues for experimentation.

Moreover, just as the *in vitro* MTS models were developed in order to improve upon existing animal *in vivo* (xenograft) models by better reflecting the *in vivo* situation, in some human cancers in silico models could eventually improve upon existing *in vitro* models by providing a better model of human *in vivo* tumor growth. We begin by emulating the *in vitro* model.

2. BIOLOGICAL BACKGROUND

Since their development in the early 1970s, researchers have used MTS to study how tumor microenvironments affect cellular growth, viability, and therapeutic response. In particular, an initial wave of research on MTS in the 1970s and 1980s extensively explored growth dynamics [2–5]. More recently, researchers have shifted towards using the MTS system as a model to study other topics in oncology, such as cell cycle regulation [9].

An MTS consists of a roughly spherical aggregate of tumor cells in suspension culture. Researchers have described MTS as exhibiting similar behaviors over a wide range of

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culture conditions: a growth curve consisting of initial exponential expansion in volume and cell number followed by growth saturation; and a concentric spatial layered structure, consisting of a thin outer shell of actively proliferating cells, an intermediate layer of viable but quiescent cells, and an inner necrotic core [10].

The *in vitro* experimental methods and growth of MTS can be briefly summarized as follows. A small aggregate of tumor cells is placed in a spinner flask containing growth medium. Oxygen is bubbled through the flask, while the contents of the flask are replaced roughly every 12-24 hours to replenish the supply of nutrients. Tumor cells consume oxygen and nutrients from the medium, and under favorable conditions, begin proliferating. This leads to an initial exponential growth phase, as the cell volume and population increase. Metabolic activity causes decreased oxygen and nutrient availability and an accumulation of metabolic byproducts toward the interior of the spheroid, creating gradients in these substances. These conditions contribute to quiescence and subsequently apoptosis and necrosis near the center of the spheroid. The necrotic cells release cytotoxic products that may inhibit tumor spheroid growth. Tumor cells near the surface of the spheroid continue to proliferate, as favorable environmental conditions are maintained via periodic replenishment of the growth medium as stated above.

3. METHODS

The agent-based model (ABM) is designed to closely approximate the experimental components and methods of the *in vitro* referent. It is created using JAS, a fine-grained agent-based modeling tool kit [14]. JAS provides a framework for the creation and execution of models, including tools for event scheduling, visualization, and IO. It utilizes third party libraries for database interactivity, statistical analysis, random number generation, plotting results, and other functions. The ABM is a discrete event model, so that while some events, such as diffusion and resource consumption, take place at every time step, other events, such as movement and shedding take place only over longer intervals.

There are four types of elements (objects) in the model: simulated tumor cells (CELLS[1]), OXYGEN, NUTRIENT, and INHIBITOR. Early versions of the model grouped oxygen and nutrients into a single category, but it was apparent that their simulations could not mimic referent data taken at varied levels of oxygen and glucose. We therefore separated oxygen and nutrients into two independent variables. The nutrient category contains both the variable glucose levels and the other, constant, nutrients within the medium. We believe that this level of abstraction is the simplest that will allow our models to represent experimental methods and mimic referent results. Similarly, all inhibitory factors resulting from apoptosis and necrosis (such as metabolic and catabolic waste products, necrotic material, etc.) are grouped into the INHIBITOR category. There are different spaces for OXYGEN, NUTRIENT, INHIBITOR, and CELLS. The contents of the OXYGEN, NUTRIENT, and INHIBITOR spaces diffuse through their environment toward sources of lower concentration. The OXYGEN and NUTRIENT are regularly replenished to initial levels in locations outside the tumor. The simulation begins with a single CELL that proliferates outward. All CELLS in the model will change state depending on the local environment, and have the potential to move, shed, proliferate, or die. Figure 1 is a screenshot of the model's JAS implementation.

3.1 Computational Spaces

Each type of element resides in its own "Computational Space." The environment of the ABM consists of overlapping layers of spaces (see Figure 1). The computational spaces for OXYGEN, NUTRIENT, and INHIBITOR consist of 2D square grids of values that range from 0 to 1, while the space for CELLS holds references to the individual CELL objects.

3.2 Diffusion

For transport of OXYGEN, NUTRIENT, and INHIBITOR, we use a discrete 2nd order approximation of the 2D diffusion equation, $du/dt = D\nabla^2 u$. This equation is discretized and solved in the 2nd order using a 2D diffusion kernel, giving values for the coefficients of diffusion from each CELL to its neighbors [17]. A single CELL will determine its new value using the equation, $u_{new} = (1 - d)u + 0.05d(4u_N + 4\,u_S + 4\,u_E + 4\,u_W + u_{NE} + u_{NW} + u_{SE} + u_{SW})$ where d is the coefficient of diffusion and u_N through u_{SW} are the values of the eight locations in the Moore neighborhood of the CELL. Because the equation is solved in the 2nd order, diagonal neighbors are less favored and an approximately circular diffusion results.

3.3 Replenishment

Every 10 simulation cycles the OXYGEN and NUTRIENT are replenished to initial levels, so that the area immediately outside the spheroid does not become depleted. Replenishment is carried out by resetting levels of OXYGEN and NUTRIENT to initial levels in every empty location that is outside the tumor. The replenishment algorithm is run more often in the simulation than is actually carried out *in vitro* in order to simulate the effects of convection and mixing currents within the system. The parameters (and their values) controlling CELL behavior during simulations are listed in Table 1. In both the *in vitro* and in silico system the area immediately surrounding the tumor spheroid does not become significantly depleted.

[1] We use SMALL CAPS to distinguish the in silico analogue of an element or feature from its *in vitro* referent.

3.4 Tumor Cells

Each CELL can exist in a number of states and has variables that represent aspects of biological cells. The ENERGY within a CELL represents the internal volume and energy stores within a referent cell. In a given cycle, each CELL will move through different states and execute different actions depending on its internal and external environment. The CELL will first determine its state, then convert OXYGEN and NUTRIENT from the environment into ENERGY, and then, depending on its state, possibly move to another location or proliferate, creating a daughter.

3.5 Tumor Cell States

Each CELL is in one of five states at each cycle (Figure 2):

- PROLIFERATE, which corresponds to early G1 phase
- PREMITOTIC, which corresponds to late G1, S, and G2
- MITOTIC, which corresponds to M phase
- QUIESCENT, which corresponds to arrested CELLS
- APOPTOTIC, which corresponds to CELLS that have undergone APOPTOTIC or NECROTIC CELL death

For each CELL, its state is determined at each time step based on the values of the CELL'S internal variables and the local microenvironmental conditions, i.e., the conditions within its location. These rules are illustrated in Figure 2.

The stochastic variable prevents CELLS from all changing states at the same time, which would result in periodic waves of growth. It also simulates the variability within certain parts of the cell cycle.

If the CELL is in the MITOTIC state it will check if mitoCounter (decremented during each turn of mitosis) has dropped below zero. Once it does, the CELL will create a daughter in a neighboring location.

3.6 Tumor Cell Energy and Metabolism

In the model, CELLS use ENERGY as an analog for changing cellular volume and chemical energy. Each CELL consumes a certain amount of OXYGEN and NUTRIENT from its local environment during a time step. Their rates of

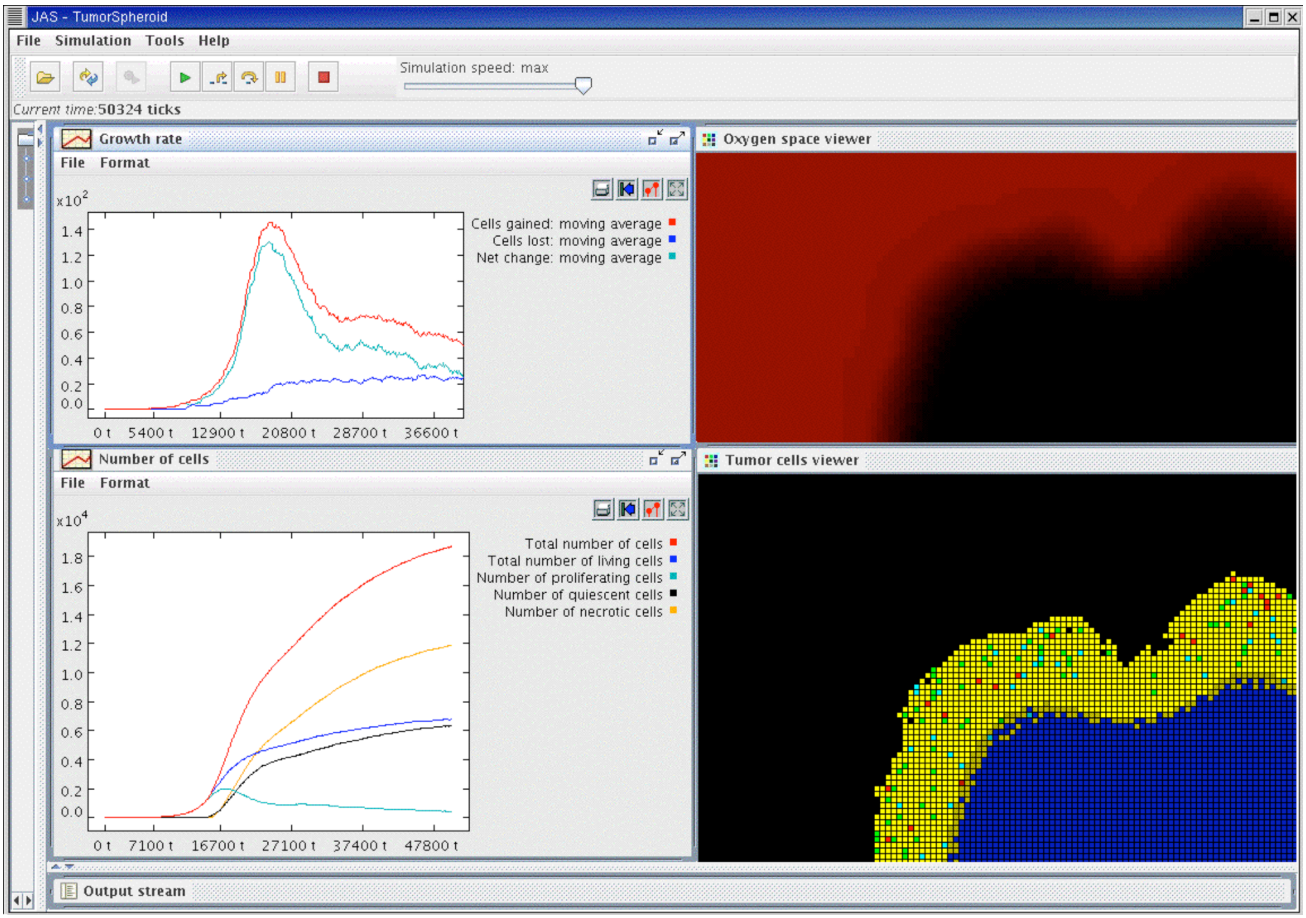

Figure 1. Screen shot from a simulation run. The upper-left quadrant of an in silico multicellular tumor spheroid (IS-MTS) is shown. Each grid location represents an individual CELL. The user can scroll to any part of the IS-MTS and click on an individual CELL to examine its internal state and variables. The color of each location is determined by the cellular state of the corresponding CELL, according to the following map: green = PREMITOTIC (late G1, S, G2 phase), light blue = MITOTIC (M phase), red = PROLIFERATE (early G1 phase), yellow = QUIESCENT (G0 phase, arrested), dark blue = NECROTIC/APOPTOTIC. The darker CELLS represent CELLS performing GLYCOLYSIS. The OXYGEN Space is also viewable, with darker values representing lower OXYGEN concentrations. The growth rate and number of CELLS are graphed in real-time during a simulation run.

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consumption are determined by CELL state. The CELLS convert the consumed OXYGEN and NUTRIENT into ENERGY, which is used for CELL survival, proliferation, and movement. The relative rates of conversion can be adjusted by the user. Under normal conditions the amount of OXYGEN and NUTRIENT consumption are fixed, but when a CELL runs out of OXYGEN it will switch from simulating respiration to simulating glycolysis, creating ENERGY purely from NUTRIENT. This process is less efficient than respiration, however, and so the amount of NUTRIENT required to produce a unit of ENERGY is multiplied by the glycoFactor parameter in Table 1.

The parameter consumeEnergySurvive specifies the amount of ENERGY that a CELL will consume each cycle in order to maintain basic function. The parameter consumeEnergyPro specifies the amount of ENERGY a CELL will consume when it is in the PROLIFERATE, PREMITOTIC, or MITOTIC state. The difference between this value and consumeEnergySurvive is the extra ENERGY that is saved up each turn toward proliferation. When a CELL goes from the PROLIFERATE state to the PREMITOTIC state it must spend an amount of ENERGY equal to prolifCost. In order to move from the PREMITOTIC state to the MITOTIC state the CELL spends ENERGY equal to mitoCost.

3.7 Inhibitor Production

Tumor cells release metabolic byproducts and one or more toxic inhibitors when they become necrotic, which act to inhibit the activity and proliferation of other cells. These factors are thought to lead to the development of growth saturation in MTS.

We combine these products into a single toxic INHIBITOR class to reflect that these products inhibit CELL activity. When a CELL dies it releases a quantity of toxic INHIBITOR into the environment, which will diffuse outward. Any CELL exposed to the INHIBITOR has a certain probability of arresting by shifting into the QUIESCENT state, which is based on its maxTox level, a unique random number calculated from the parameters maxToxLow and maxToxHigh. Each CELL has a different maxTox in order to prevent all CELLS within the tumor spheroid from becoming quiescent at the same time.

3.8 Tumor Cell Shedding

CELLS within a certain area near the center of the grid are restricted from shedding, which approximates the *in vitro* experimental methods, in which MTS are placed in the spinner flask only after reaching a certain size. For each CELL outside of this area, the following algorithm is used to implement shedding. We first check to see if the CELL is on the outside or the inside of the tumor. Only CELLS on the outside of the tumor can be shed. We then find the number of CELL neighbors, only allowing CELLS with fewer than three neighbors to be shed. The probability of shedding is lower the greater the number of neighbors, and lower if the CELL is not in the MITOTIC state [15]. The CELL will be shed if a random number $p_{(0,1)} <$ probShed.

Table 1. Parameters for in silico simulation runs. All listed parameters can be changed, but it is only necessary to change a small number for the experiments run within this report.

Parameter	Value / Range	Description
Environment		
oDiffuse	0.7	OXYGEN diffusion rate
nDiffuse	0.5	NUTRIENT diffusion rate
tDiffuse	0.4	Toxic INHIBITOR diffusion rate
startOx	0.03–0.12	OXYGEN concentration empty locations are replenished to
startNut	0.06–0.5	NUTRIENT concentration empty locations are replenished to
Tumor Cell		
minOx	0.0	Minimum level of OXYGEN required for CELLS to survive
maxOx	0.01	Minimum level of OXYGEN required for CELLS to proliferate
minNut	0.0	Minimum level of NUTRIENT required for CELLS to survive
maxNut	0.02	Minimum level of NUTRIENT required for CELLS to proliferate
maxToxLow	5.0×10^{-5}	The low end for CELL sensitivity to the toxic INHIBITOR
maxToxHigh	0.1	The high end for CELL sensitivity to the toxic INHIBITOR
consumeEnergyPro	3.0×10^{-4}	ENERGY consumed by proliferating CELLS; any extra is saved
consumeEnergySurvive	2.75×10^{-4}	Base ENERGY consumed by CELLS to survive
deadOutputTox	0.3	Amount of INHIBITOR released by CELLS when they die
prolifCost	0.01	ENERGY cost for a CELL to move from PROLIFERATE to PREMITOSIS
mitoCost	0.01	ENERGY cost for a CELL to move from PREMITOSIS to MITOSIS
mitoTurns	100	Number of time steps a CELL spends in MITOSIS
premitoTurns	750	Number of time steps a CELL spends in PREMITOSIS
oxToEnergy	1.0	Amount of OXYGEN consumed to produce a unit of ENERGY
nutToEnergy	1.0	Amount of NUTRIENT consumed to produce a unit of ENERGY
glycoFactor	10.0	During GLYCOLYSIS NUTRIENT consumption increases by this factor
stateProbProlif	0.05	Probability a CELL will PROLIFERATE in semi-favorable conditions
stateProbApop	0.2	Probability a CELL will be APOPTOTIC in unfavorable conditions
probProlif	5×10^{-3}	Probability a CELL will move from PROLIFERATE to PREMITOTIC to MITOTIC
probShed	0.35	The seed probability a CELL will be shed
probMove	0.15	The probability a CELL will move
stateTime	100	Minimum time steps a CELL will stay in a state after transitioning
eventDelay	100	Number of time steps that pass between certain events

3.9 Tumor Cell Dynamics

CELLS are able to move occasionally, and when they do so they will move into the neighboring location with the highest levels of OXYGEN and NUTRIENT. The algorithm for movement is as follows:

The CELL will first determine if any neighboring locations are empty, in which case it will pick the best (defined by having the highest OXYGEN and NUTRIENT) potential location to move into. If all neighboring locations are occupied, the CELL will find the best occupied location and choose that as the potential location. If a random number $p_{(0,1)} <$ probMove, the CELL will move into the selected location. The variable probMove is multiplied by four if the location is an adjacent rather than diagonal neighbor in order to preserve the 2nd order geographic locality. If the selected location is occupied, the CELL will swap positions with its chosen neighbor. This strategy represents simplifying assumptions that allow location change in densely packed CELL aggregates.

Once a MITOTIC CELL is selected to proliferate, it will iteratively find the closest open grid location and proliferate toward that location. In order to do this it will push the CELLS between it and that location one step in the direction of that location. The algorithm does not take into account the levels of NUTRIENT or OXYGEN in the locations, but assumes that intercellular forces will be the most important factor in deciding in which direction the new CELL will appear.

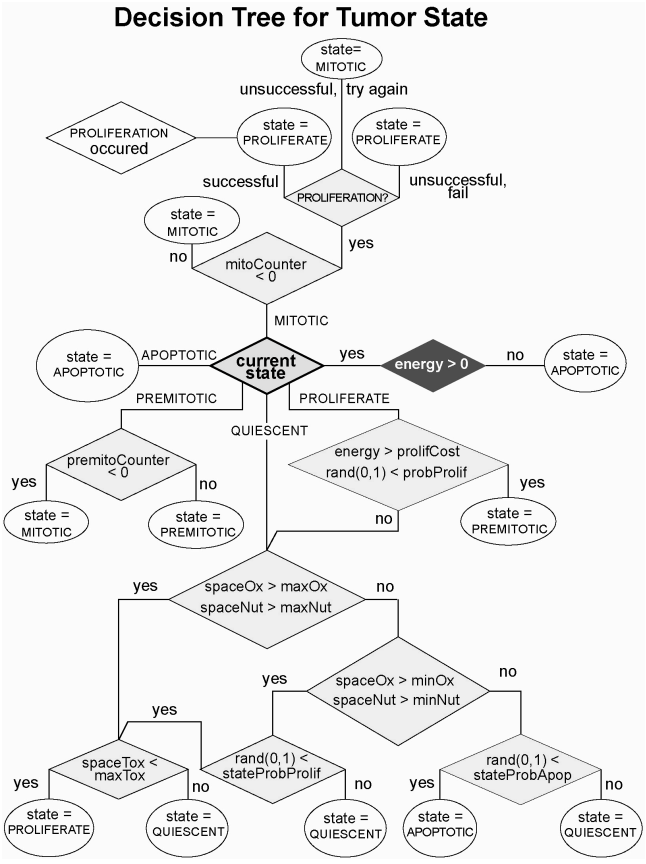

Figure 2. Decision tree for tumor state: The path that a simulated tumor cell takes to determine its state during a given time step is shown. The CELL begins by checking if energy > 0, and continues by checking the current state. Random numbers are generated using the CERN Colt implementation of MersenneTwister. During each cycle that a CELL is in MITOSIS or PREMITOSIS the values mitoCounter or premitoCounter are decremented. If the CELL is able to successfully PROLIFERATE it will produce a daughter in a neighboring location.

4. SIMULATIONS & RESULTS

4.1 Basic Growth Curve

Sutherland et al. observed that there are three unique phases of *in vitro* tumor spheroid growth: an initial exponential phase, a linear phase, and a final logarithmic or saturation phase [6]. We observed three similar phases in the growth of in silico spheroids. The initial phase occurs while CELLS have unlimited access to NUTRIENTS and OXYGEN and are not too restricted by the presence of neighboring CELLS. The linear phase occurs once the CELLS toward the center of the spheroid begin to run out of NUTRIENT or OXYGEN and lasts until the saturation phase begins, which occurs when enough INHIBITOR diffuses out from the dying CELLS and accumulates within and around the tumor to slow the growth rate of CELLS outside the core. These phases qualitatively align both with the Gompertz growth function and with a compilation of exponential, linear, and logarithmic functions.

Some early inductive models of tumor spheroid growth [7] were able to successfully describe the exponential and linear phases, but were not able to produce the saturation phase. Other models [8] were able to produce all three phases. Our model also simulates the three phases (Figure

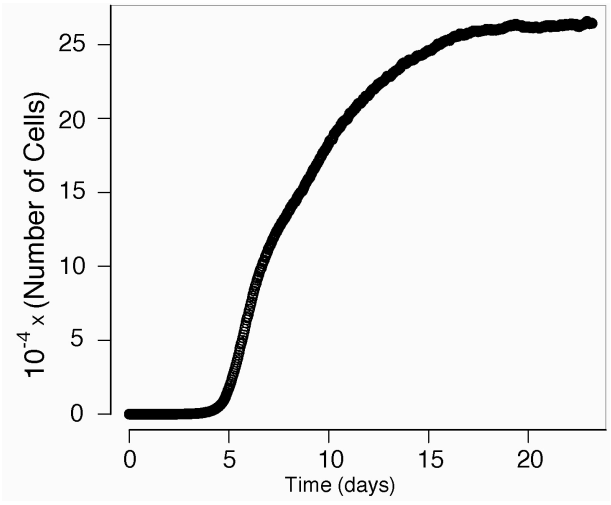

Figure 3. Results of an in silico simulation: OXYGEN = 0.12 and NUTRIENT = 0.5. The simulation is scaled so that 23.2 *in vitro* days = $1x10^6$ time steps and 1 in silico CELL = 0.25 *in vitro* tumor cells.

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3) within certain ranges of parameters. Notably, if the diffusion rate of the necrotic INHIBITOR is slower than the outward proliferation of new CELLS, the necrotic INHIBITOR will never be able to slow the outward growth and saturation will not occur. In addition, if the CELLS are not sensitive enough to the necrotic INHIBITOR, then only a few of them will become QUIESCENT and the spheroid as a whole will continue to expand outward. Finally, if the rate of shedding is too low then shedding will not create sufficient CELL loss to counteract the CELL growth that occurs during the saturation phase. None of these parameters, however, map to *in vitro* factors or variables that can be either changed or controlled. As such, even though the model is sensitive to changes within the above-cited parameters, once they are fixed within certain acceptable ranges, the ABM becomes robust. The user can vary other parameters, such as the initial concentration of OXYGEN and NUTRIENT, without changing the system-level characteristics of the ABM. Although a spheroid might grow slower or faster and saturate at a smaller or larger final size, it will overall continue to produce the three basic phases of growth that are characteristic of the *in vitro* phenotype

4.2 Reaching Saturation

One of the necessary conditions for reaching growth saturation is that there be a balance between CELL gain and loss. Within the *in vitro* system, balance is thought to be achieved from a combination of proliferation, tumor cell shedding, and apoptotic/necrotic cell death. Our in silico spheroid achieves a similar balance using the same CELL gain and loss mechanisms.

A model can often say more by what it does not contain rather than what it does contain. Our model is notable because it is able to mimic *in vitro* results even though it lacks two yet-to-be verified hypothetical attributes that are included in other more traditional models. The first is an inward driving "chemotactic force": Dormann and Deutsch made the assumption that a "chemotactic force" would motivate tumor cells to move toward the center of the spheroid, thus providing a mechanism for the control of unlimited growth [11]. In our model, control of spheroid expansion is caused by the slowing growth rate and an increasing rate of CELL shedding. The second is an external physical "compression force": Drasdo and Hohme assumed that this force would cause tumor cells to grow more slowly and somehow to become stable [12]. Although there is evidence for physical compression forces within both *in vivo* and *in vitro* cultures grown in 3D matrix cultures, no evidence exists suggesting that cells are compressed within suspension cultures. We hypothesize that the cells stop growing because of some other process.

In this report we explore the effects of the necrotic inhibitor, a concept that has not been closely examined in the recent literature. LaRue et al. hypothesize that the necrotic factors contribute to the release of cytokine inhibitors, but they do not discuss what form the necrotic factors may take [13]. Our experiments lead us to posit that *in vitro,* the necrotic material must diffuse out of the spheroid in such a way that it can make contact with cells even at the outer edges of the spheroid. In the ABM, if this were not the case, the CELLS at the outer edge would never stop growing and the tumor would not reach saturation. Also, we assume that CELLS' susceptibility to the necrotic material varies, since otherwise the uniform distribution of necrotic material would create a uniform distribution of growth arrest.

4.3 Growth During Varying Culture Conditions

We conducted experiments at varying levels of NUTRIENT and OXYGEN over 100,000 simulation cycles, which corresponded to 23.2 days of growth in the *in vitro* model [3]. The results are shown in Figure 4. Overall, the similarity between the *in vitro* and in silico data is acceptable. Recall that an equation-based model is fit to just one set of conditions at a time. The growth curves for high OXYGEN–low NUTRIENT and low OXYGEN–high NUTRIENT runs are particularly close to the referent data. At higher levels of OXYGEN and NUTRIENT, the ABM displays a much faster initial growth phase followed by a more dramatic saturation. This is most likely due to the CELLS' unlimited growth potential when ample NUTRIENT and OXYGEN are available, a potentially unrealistic assumption made to keep things simple. At the lowest values of NUTRIENT and OXYGEN the model predicts a slightly larger saturation size, though the overall level is lower than at any of the other simulation conditions. Under such stressed *in vitro* conditions, other mechanisms, which are absent or insignificant at the other conditions, may have influence. A planned next step is to quantify the similarity between *in vitro* and in silico experimental results through statistical analysis and similarity measures [16].

An earlier ABM contained a simple representation of

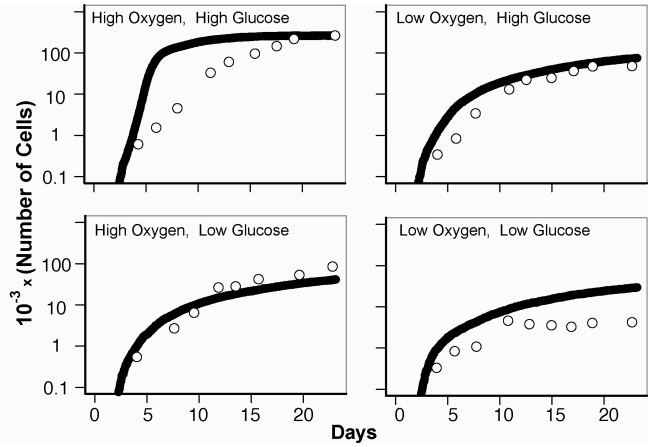

Figure 4. Spheroid cell number is plotted vs. days of growth in four different culture conditions. Heavy lines represent in silico simulations. Circles represent *in vitro* data from [3]. High in silico NUTRIENT corresponds to 16.5mM *in vitro* glucose, low to 0.8 mM; high in silico OXYGEN corresponds to 0.28 mM *in vitro* oxygen, low to 0.07 mM.

metabolism that did not consider glycolysis. In that model, when a CELL ran out of either OXYGEN or NUTRIENT it could no longer create ENERGY and began to die. When we attempted to match the *in vitro* results in Figure 4 we discovered that the high OXYGEN-low NUTRIENT, low OXYGEN-high NUTRIENT, and low OXYGEN-low NUTRIENT conditions resulted in identical behavior. The *in vitro* data in Figure 4 clearly invalidated that model. In order to address this issue we created the current version of the ABM with a more detailed representation of metabolism. This representation is capable of simulating behaviors that are more realistic when NUTRIENT and OXYGEN are varied. In silico, at high levels of OXYGEN, CELLS can survive using efficient cellular respiration. At high NUTRIENT levels, the CELLS can compensate for a lack of OXYGEN by going into GLYCOLYSIS. At low levels of NUTRIENT and OXYGEN, the spheroid has insufficient NUTRIENT to compensate for low levels of OXYGEN, and thus grows slower and saturates at a smaller size.

4.4 Spheroid Diameter at Onset of Necrosis

Figure 5 is a plot of spheroid CELL number at saturation vs. diameter at onset of NECROSIS, for eight different combinations of OXYGEN and NUTRIENT. The data can be directly compared to the *in vitro* data from Freyer and Sutherland [3], which demonstrated a linear relationship between spheroid diameter at the onset of necrosis and cell numbers at saturation. Freyer and Sutherland considered the data strong evidence that oxygen and nutrient concentration gradients regulate the onset of necrosis, and that necrosis regulates tumor spheroid size at saturation. They argued that this result provides evidence for the existence of a necrotic factor that inhibits cell growth. They presented their data in two graphs, each at different oxygen concentrations, and we have done the same.

For these conditions, we assumed that NECROSIS is detectable after 50 CELLS have become NECROTIC. In order to calculate the diameter at the onset of NECROSIS, we measured the number of LIVING and NECROTIC CELLS and found the radius of the cross section using the formula for the area of a circle. To extrapolate the size of the sphere at saturation, we calculated the radius of the cross section and then used the formula for the volume of a sphere to calculate the size of the NECROTIC core and the size of the entire spheroid. We then subtracted the size of the NECROTIC core from the size of the entire spheroid to find the number of LIVING CELLS. This number was plotted against the diameter of the spheroid at saturation for eight different values of NUTRIENT and OXYGEN. A linear relationship resulted, similar to that reported by Freyer and Sutherland [3]. This result is significant because it demonstrates that the ABM is capable of producing behaviors that are not specifically targeted. It also provides evidence that in silico tumor spheroid growth saturation is primarily regulated by the toxic INHIBITOR, mirroring the *in vitro* conditions. This is a precondition for testing *in vitro* hypotheses surrounding the necrotic material; the in silico model must display similar results in this domain in order to allow for the simulation of further experiments involving growth saturation. The parameters used for the production of this result are the same as the parameters used to produce the growth curves in Figure 4, mimicking *in vitro* conditions. The exception is that eight different combinations of OXYGEN and NUTRIENT were used instead of four. We found the new NUTRIENT values (0.0943 and 0.225) by mapping the ratios of glucose from the *in vitro* experiments to the chosen low and high NUTRIENT levels in our in silico simulation.

5. DISCUSSION

A near-term goal of this project has been to create a model that reasonably represents the behaviors of an *in vitro* MTS while being composed of separate individual CELLS. The process of further developing the ABM will expose

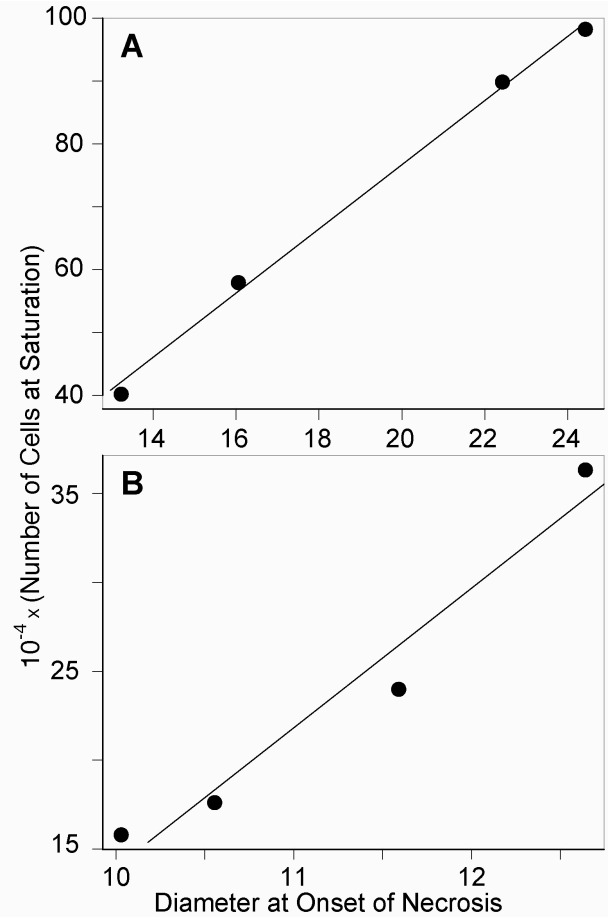

Figure 5. Spheroid cell number at saturation vs. diameter at onset of necrosis for varying levels of NUTRIENT and OXYGEN. Upper graph: OXYGEN = 0.12, NUTRIENT = 0.06, 0.0943, 0.225, 0.5. Lower graph: OXYGEN = 0.03. Diameter is in cell widths.

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gaps in our understanding of tumor spheroid behavior and help lead experimentalists in new and novel directions. There are many unanswered questions, including the exact action of the necrotic inhibitors and the mechanism of volume loss within the MTS. Our simulation produces plausible results even though it lacks two yet-to-be verified hypothetical attributes that are included in other models, as mention in section 4.2. We anticipate that the more mature offspring of in silico MTS could become virtual experimental vehicles for exploring ideas for novel treatments, thus giving researchers guidelines for selecting candidate therapies for further study, while providing student researchers a low cost, rapid feedback environment to hone their scientific questioning and experimental design skills.

The spheroid model discussed here is—necessarily—a work in progress. It successfully demonstrates some of the key behaviors of *in vitro* MTS. It mimics the general structures of MTS, as well as the patterns of relative time course of spheroid growth and eventual saturation. When exposed to varying levels of NUTRIENT and OXYGEN it reacts in ways that are qualitatively similar to *in vitro* MTS behaviors. It demonstrates a linear relationship between the diameter at the onset of necrosis and the eventual saturation size. Our plan for moving forward is to selectively and incrementally increase the variety of data and observations in the targeted set of phenotypic attributes. These may include data from *in vitro* experiments dealing with cell movement, cell cycle percentages, and oxygen/glucose depth profiles. We also plan to assess specific hypotheses about the source and effects of the necrotic material. Each new addition typically invalidates some aspect of the current model's behavior. The task then becomes to iteratively improve the ABM to obtain acceptable and increasing overlap between in silico and targeted phenotypic attributes. This process is expected to increase model detail, realism, and accuracy, and thereby increase model usefulness as an object of research and experimentation.

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