Understanding Emergent Biological Behaviors: Agent Based Simulations of *In vitro* Epithelial Morphogenesis in Multiple Environments

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1.1 Introduction

To understand how molecular level events are causally linked *in vitro* to the emergent, phenotypic attributes of *in vitro* systems, we need in silico analogues suitable for experimentation: they need to exhibit properties and characteristics (PCs) that overlap in useful ways with those of the *in vitro* referent. We report significant, early progress toward that goal for cultured MDCK [O'brien 2002] and related cells.

The *in vitro* morphology of MDCK cells is environment dependent. In 3D embedded cultures, cyst formation is the dominant morphological characteristic. O'Brien et al. believe that cyst formation is driven by each cell's pursuit of three types of membrane surfaces, called free, lateral, and basal [O'brien 2002]. That drive is apparently intrinsic. Epithelial cells respond to interactions with components of their environment, with other cells via cadherins and other cell-cell adhesion molecules, with matrix via integrins and other molecules, and with free surface via apically-located integrins, cilia, and flow sensing. Our plan has been to bring these mechanisms into focus iteratively by building and validating detailed analogues beginning with the one presented here: a four-component, discrete event, discrete time, and discrete space analogue. It is simple yet it exhibits a rich in silico "phenotype." It is capable of recapitulating in silico key morphological attributes [Hall 1982, Wang 1990a]. It simulates absolute, error-free mechanistic control and regulation through many generations. Error-free control is thought not to be characteristic of biological systems. We relaxed that control by providing a stochastic option to follow (or not) a rule each time the option arose. Even modest relaxation of some rules led to aberrant simulated growth characteristics that, in some cases, were similar to *in vitro* phenotypes somewhat characteristic of pre-cancerous states. The results demonstrate a potentially powerful means of gaining deeper insight into how system level phenotype emerges from orchestrations of lower level events.

1.2 Methods

To avoid confusion and clearly distinguish *in vitro* components from corresponding simulation components, such as "cells," "matrix," "cyst," and "lumen," we use small caps when referring to the latter. We use the synthetic modeling method [Ropella 2005a,b, Steels 1995] because it is especially suitable for representing spatial and discrete event phenomena. It is ideally suited for assembling analogues from components and exploring the resulting behaviors.

1.2.1 Model Specifications and Framework

We used MASON (cs.gmu.edu/~eclab/projects/mason/), an optimized, extensible Java-based toolkit. We discretized space, time, and all events. The software and executable applets are available at <http://128.218.188.102:8080/growthmodel/index.php>. We identified wet-lab experiments and observations that could be directly compared with simulated output. Our conceptual model is that epithelial cells follow innate mandates. Each cell responds to stimuli based on its need to comply with all of these mandates. The observed repertoire of behaviors is hypothesized to be a consequence of stimulus-response mappings between inputs and outputs at a cell level "interface." The first task was to specify initial spatial and temporal resolutions. We treated each cell and similar sized unit of environment as single (atomic) objects. We assumed that the cell accepts stimuli and later, when appropriate, responds resulting in a change in state. We assumed that a repertoire of stimulus-response sequences precedes a state change, and that response times for different state changes can differ. We used one simulation cycle to represent all state changes. Events such as macromolecular binding or signaling are below this level of temporal resolution. Because events in two different *in vitro* systems can proceed at different paces, one simulation cycle can map to different intervals of wet-lab time.

Processes such as changes in cell shape or in local matrix organization are not ignored; they are below the level of spatial resolution. Their contribution is conflated into axioms[1] (illustrated in Fig. 2) and the components. A detailed stimulus-response mapping between inputs and outputs, such as initiation of apoptosis, are below the levels of temporal and event resolution. We assume that they can be represented collectively by the axioms. CELL behaviors are independently scheduled; they are executed in sequence. The logic governing CELL behavior is embedded within the CELL (the CELL is an agent).

Four simulated environments represent the referent environments. Each consists of a 2D (or 3D) hexagonal grid, typically 100 x 100. Just one of three different types of components is assigned to each grid point: MATRIX, FREE SPACE, or CELL. MATRIX represents a cell-sized region of culture medium that contains matrix. FREE SPACE represents a similar sized region of cell-free or matrix-free medium. An *in vitro* surface culture is represented by a grid in which there is an upper region of FREE SPACE, and a lower region of MATRIX. At simulation initiation, a single CELL is placed at the CELL–FREE SPACE interface in place of a FREE SPACE. An embedded culture is represented by a grid having MATRIX in all locations. To initiate a simulation, one or more CELLS replace MATRIX. Suspension culture is represented by a grid in which FREE SPACE is assigned initially to all locations. A simulation is initiated by having one or more CELLS replace FREE SPACE. Last,

[1] *Axiom* emphasizes that computer programs are mathematical, formal systems and the initial mechanistic premises in our simulations are analogous to axioms in formal systems. For our analogues, an axiom is an assumption about state change that is assumed true for the purposes of further analysis or deduction, and for developing the analogue system.

simulation of an overlay or sandwich culture starts with a single horizontal monolayer of CELLS bordered above and below by a region of MATRIX.

The only components in the simulation that schedule events are CELLS. We assumed that all behaviors are influenced by only the immediate environment. Each CELL has a new event scheduled for it at the next simulation cycle, and it then executes a program (Fig. 2) and a decision process (Fig. 3). For each neighborhood arrangement, there is only one action option.

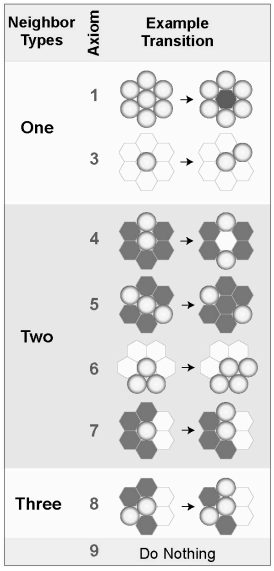


Figure 2. Illustrations (2D hexagonal grid) of the axioms that govern a CELL'S state transition during any simulation cycle. Dark hexagons: FREE SPACE; circles with gradient shading: CELLS; and white hexagons: MATRIX. Axioms are organized by the number of object types in the local neighborhood: one or more of CELL, MATRIX, and FREE SPACE. Axiom 2 (die if all neighbors are FREE SPACE) is not shown because it was not used. Each axiom defines a mapping from an initial to a final state for the center CELL making the decision. Decision options (sketched in Fig. 3) are based on the arrangement of three object types adjacent to the CELL. Multiple locations can meet the axiom's requirement for daughter CELL placement. When that occurs, one is selected at random. When none of the conditions of the first eight axioms is met, Axiom 9 applies: does nothing.

1.2.2 Axiom Development and Experimentation

In order to allow for unanticipated behaviors in each environment, we assumed that a cell's action in a particular environment is independent of its actions in others and from any past action. For a hexagonal grid containing arrangements of three different components (CELL, MATRIX, and FREE SPACE), the 726 possible neighborhood arrangements were reduced when mirror images are assumed equivalent, and because several arrangements are identical after axial rotations. We classified the remaining arrangements based on their perceived similarities with respect to overall cell behavior. We arrived at a set of arrangements in which cell behavior could be functionally distinct. Guided by specific literature observations of *in vitro* systems, we assigned a prediction as to how an epithelial cell would behave in each. For the remaining cases, we assumed that epithelial cells desire to maintain existing surfaces, and generate additional environment types, in a pursuit of three surface types [O'brien 2002]. Merging similar behavior predictions resulted in simplified collections of axioms (Fig. 2) and the decision sequence (Fig. 3).

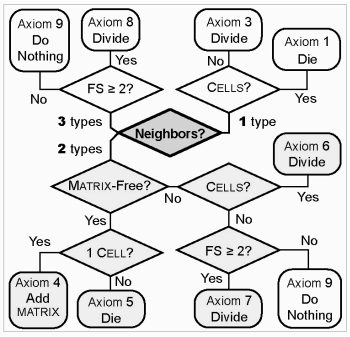


Figure 3. A diagram of the decision sequence used to select the axiom that specifies a state change for a CELL. Decisions are made based on types of neighboring objects (see Fig. 2). FS: FREE SPACE.

During each simulation cycle, each CELL uses the decision tree to select one of four actions: do nothing, die, add MATRIX, and divide (two axioms manage daughter placement differently). First, each CELL determines if it has one, two, or three types of neighbors. If there is only one type, then follow either Axiom 1 or 3. Two types: follow either Axioms 4, 5, 6, or 7. Three: follow Axiom 8. If 1-8 does not apply, do nothing. The if–then axioms are: 1) only CELL neighbors: die; 2) only FREE SPACE neighbors: die (does not apply for these simulations); 3) only

MATRIX neighbors: divide; 4) neighbors are FREE SPACE and just one CELL: add MATRIX between self and that CELL; 5) neighbors are FREE SPACE and ≥ two CELLS: die; 6) neighbors are ≥ one CELL and MATRIX: divide and replace a MATRIX with daughter to maximize her CELL neighbors; 7) neighbors are MATRIX and ≥ two FREE SPACE: divide and place daughter in a FREE SPACE to maximize her MATRIX neighbors; and 8) three types of neighbors with MATRIX neighbored by two adjacent FREE SPACES: divide and place daughter in a FREE SPACE to maximize her MATRIX neighbors.

To insure that results were nondeterministic, random number generator seeds and the scheduling of events were changed for each simulation. For analysis of CYST formation, experiments were conducted in simulated embedded culture: typically, one-to-two hundred cycles, with up to two hundred complete repetitions. Each cycle represents approximately six to nine hours *in vitro*. Images of each completed simulation were collected for subsequent comparison. Cell numbers per CYST were saved. In simulated surface culture, we ran 50 simulations. Each used 3D square grids (2 x 100 x 100): first, the bottom section was filled with MATRIX. A CELL was then placed at the center of the top 1 x 100 x 100 grid. *In vitro* surface culture data (days 0–5) was available [Taub 1979] for comparison. For this growth environment, we assumed each simulation cycle corresponds to 20.4 hours of *in vitro* time.

To examine the importance of the orientation of daughter placement on simulated morphology, Axiom 8 was changed to allow for placement of the daughter into any neighboring FREE SPACE location. As a strategy to get a variety of CYST sizes in simulated suspension culture and to simulate the effect of altered matrix production, Axioms 4 and 5 were replaced with one that specified the placement of MATRIX at a neighboring FREE SPACE position with a maximal number of CELL neighbors.

A probabilistic option was added to Axiom 1 to allow CELL survival in CELL-only environments. When a rule event is executed and the CELL is in a CELL-only environment, there is a probability that the cell will DIE. That probability is a parameter value shared by all of the CELLS in a particular simulation run. At each event, a CELL simulates sampling from a uniform distribution using random number generation. CELL numbers were recorded at each step, for fifty steps.

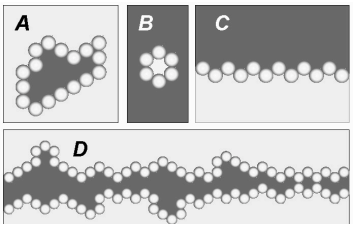


Figure 4. Example output from the four simulated environments. Spheres represent CELLS; black space represents FREE SPACE or simulated internal lumen space; white space represents MATRIX. **A**: stable CYST formation in simulated embedded culture; **B**: stable CYST formation in simulated suspension culture; **C**: MONOLAYER formation in simulated surface culture stable; **D**: LUMEN formation in simulated overlay culture. These results match *in vitro* results [Hall 1982, Wang 1990a]

1.3 Results

1.3.1 Targeted Phenotypic Attributes

MDCK cells grown on collagen I generate a simple monolayer that covers the entire surface [Madin 1975]. Cell division takes place on the outer edge [Nelson 2005]. Analogue simulations successfully represent these attributes (Figs. 4C & 5A). Growth in simulated surface culture begins with an initial exponential growth phase that lasts for three simulated days. It closely matches published observations of [Madin 1975] (Fig. 5A). Overlay of a monolayer with Collagen I induces a morphological response [Schwimmer 1995] that results in formation of flat lumen

spaces completely surrounded by cells [Hall 1982]. The events include migration, division, apoptosis, and shape changing. Simulated overlay culture resulted in similar structures (Fig 4D). MDCK cells grown in type I collagen (embedded culture) exhibit clonal growth in three stages [Wang 1990b]: 1) repeated rounds of cell division during the first two days of culture, followed by 2) the formation of a central lumen accompanied by an increase in cell numbers and cyst diameter between day two and seven. 3) Thereafter, cyst size plateaus with little apparent change thereafter. The data in Figs. 5A & B show similar patterns. Example CYSTS are provided in Figs. 4A & 6A.

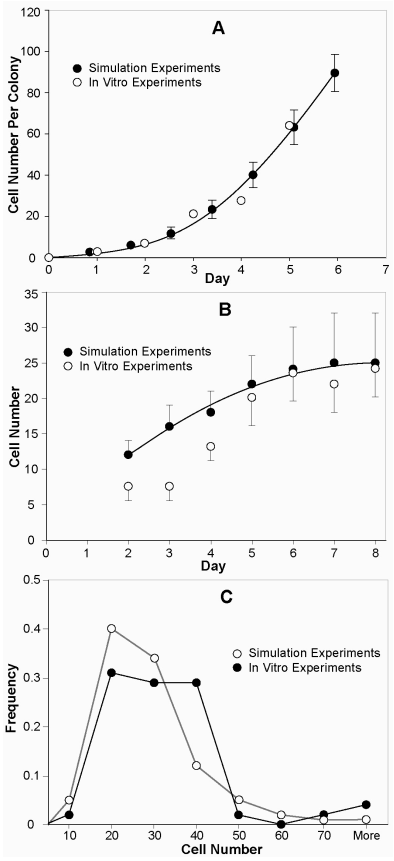


Figure 5. Comparisons of data from in silico (n = 50) and *in vitro* experiments, assuming a mean CELL division time of 20.4 hours: **A**: CELL numbers per COLONY in simulated surface culture; **B**: CELL numbers per CYST in simulated embedded culture, where two simulation cycles corresponds to 1 simulated day; **C**: comparison of *in vitro* cell numbers per cyst cross-section after ten days in embedded culture with CELL numbers per CYST cross-section after twelve simulation steps.

In simulated embedded culture MORPHOGENESIS demonstrated five similarities with *in vitro* [Wang 1990a]. a) LUMEN formation occurred after 2–2.5 simulated days; it occurred *in vitro* by as early as day two. b) CYST expansion was arrested (Fig. 5B). c) Stable CYSTS contained central FREE SPACE lined by a single layer of CELLS. d) CELL division and CELL death continued after the initiation of LUMEN formation [Lin 1999] (Fig. 5C). e) There was variation in CELL numbers per mature CYST, similar to *in vitro* observations (Fig. 5C).

The fourth targeted attribute is formation of cysts in suspension cultures. Cystogenesis *in vitro* begins with the formation of aggregates of two to ten cells. Thereafter, over approximately ten days, an "inverted" cyst (Fig. 4B) forms, expands, and then stabilizes. During the process, basement membrane components accumulate in the lumen and line the inner surface of the cyst [Wang 1990a, Nelson 2005]. We limited MATRIX production in the simulation to one situation: Axiom 4. This abstract behavioral specification is sufficient to enable formation of small, stable CYSTS (Fig. 4B), but fails to represent any of the other characteristics.

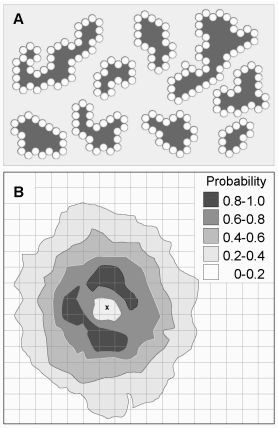


Figure 6. CYST properties. ***A***: examples of CYSTS formed in simulated embedded culture. The selection illustrates the range in CYST size and shape. ***B***: the contour plots show the probability of CELL location, relative to CYST center (x), for a stable CYST formed in simulated embedded culture. The location of each CYST'S "center" was defined as the average grid location for the CELLS forming

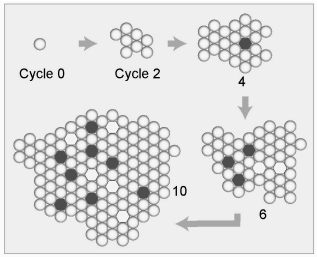
that CYST. Relative to each CYST'S center, the frequency of finding a cell at each grid location was determined for two hundred embedded culture simulations that each ran for 100 steps (all CYSTS stabilized). The contour plot shows that averaged over many simulations, CYSTS tend to be circular with one LUMEN.

There are three noteworthy differences between *in vitro* and in silico behaviors in embedded culture. There is occasional expansion of cysts [Wang 1990a] as consequence of division by cells that have already made contact with three environments. There is no corresponding behavior during simulations, because the operative mechanisms are below the level of resolution of this analogue. The second difference is the variability in CYST shapes. Some stable CYSTS are quite aspherical (wrinkled and puckered). Figure 6A contains a representative set. Such shapes are rarely observed *in vitro*. Given positive pressure differentials between the inside and the outside of cysts, any homogeneous luminal space would tend to become spherical. We can simulate the consequences of such pressure differences (but have not). A consequence would be that all CYSTS would be more circular. Because *in vitro* data is lacking, we elected not to include such effects. The contour plots in Fig. 6B shows, on average, that current in silico cysts tend to be circular. A third difference is that in embedded culture *in vitro* not all cell clusters form cysts. A few masses have no lumens at all; for this there is no corresponding in silico behavior.

1.3.2 Experimentation and Exploration

Cells in epithelial tumors appear less polarized [Thiery 2002]. We simulated an aspect of polarity loss by disrupting the directional CELL placement in Axiom 8 so that daughter CELLS could be placed in any FREE SPACE. The result was continued CELL proliferation resulting in amorphous combinations of CELLS, MATRIX, and FREE SPACE. That, combined with significant CELL removal due to Axiom 5, led to a proliferative, amorphous attribute in all four environments reminiscent of cancerous growths. Also of interest are observed behaviors that were not part of the original set of targeted attributes. For example, does an inverted CYST in simulated embedded culture form a normal CYST? Stable inverted CYST was placed in simulated embedded culture, and the simulation was re-started. Because of proliferation and CELL death in silico, CYST inversion and stabilization does occur. However, the *in vitro* evidence indicates that proliferation and cell death are not typically required [Wodarz 2002, Chambard 1984].

Figure 7. Screen shots taken during exploratory simulation of the consequences of liberalized matrix production: the sequence shows the effect of altered MATRIX production rules on CYST formation in simulated embedded culture. Cycle 0 is addition of one normal cell to a MATRIX-filled the grid. Alteration of Axioms 4 and 5 to allow for de novo MATRIX production in any environment consisting of FREE SPACE and CELL neighbors leads to a distinct pattern of unstable growth with MATRIX and FREE SPACE production within clusters of CELLS. CYST formation and growth arrest are not observed.



A limitation of the current model is that it failed to generate a variety of CYST sizes in simulated suspension culture. As a strategy to get a variety, we liberalized MATRIX production: we replaced Axioms 4 and 5 with a axiom that allowed for MATRIX production in any environment consisting of FREE SPACE and CELL neighbors (possibly by representing a mechanistic alteration caused by epigenetic phenomena). The change, however, did not solve the problem. Interestingly, it did result in the unanticipated behavior illustrated in Fig. 7: cellularization and MATRIX production in the center of growing clusters (Fig. 7). This axiom change produced

a hyper-proliferative, cancer-like response solely due to aberrant MATRIX production. No *in vitro* experiments have explored such behavior.

Reduction in the probability of cell death because of following Axiom 1 resulted in an increase in the number of cells found in the structures formed in simulated embedded culture (Fig. 8) but not in uncontrolled growth. The results were unexpected: a gradual shift from formation of stable structures to unregulated growth was judged a possible outcome. Application of Axiom 1, as infrequently as 1% of the time, resulted in formation of stable (yet large) structures. Furthermore, the morphology resulting from different axiom application probabilities was different, possibly analogous to events in pre-cancerous growths. Such results suggest that tight control and regulation by currently unknown mechanisms is essential for *proper* growth and morphogenesis.

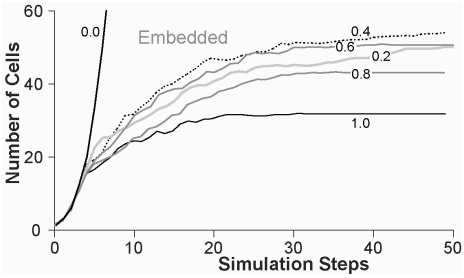


Figure 8. Consequences of stochastic application of Axiom 1 in simulated embedded culture (n = 50). In addition to p = 1.0, five probabilities were tested: 0.8, 0.6, 0.4, 0.2, and 0.0. When p = 0.4, Axiom 1 was followed 40% (average) of the time there was an opportunity to apply it. When p = 0.0, Axiom 1 was not applied. The plateau level, the number of cells in the final, stable structure, was highly dependent on the early form of that structure. Consequently, variance is large.

1.4 Discussion

Our goal was to instantiate in silico an analogue of *in vitro* cell morphogenesis based on the sensing and production of three environment components. We achieved that goal. The analogue structures mimic those formed *in vitro*: stable cyst formation in embedded culture, inverted cyst formation in suspension culture, and lumen formation in overlay culture. When selected axioms are relaxed, the targeted PCs are not achieved. Instead, cancer-like growth is observed. The observations help build the case that the approach used can be useful in identifying new plausible epigenetic or genetic mechanisms that could contribute to cancer-like growth.

The relationship between an in silico analogue and its *in vitro* referent can become similar to the relationship between an *in vitro* model and its referent, typically a feature within a patient. The *in vitro* model is a simplified abstraction of the *in vivo* target. Their two sets of PCs are intended to overlap in specific, scientifically useful ways. The region of overlap is the validity of the *in vitro* analogue. The extent of overlap relates to accuracy. There will also be large non-overlapping regions. A similar relationship is feasible between in silico analogues and their *in vitro* referents.

Although not represented explicitly in the current analogue, polarization is implicit in Axioms 6–8. Polarization *in vitro* likely represents a different cell class, enabling a different response to a given environmental perturbation. A new analogue that includes a separately validated class representing polarized cells can be validated against the current, acceptable analogue. The ability to produce and observe many different PCs from a relatively simple model makes it clear that more needs to be done to systematically expand the *variety* of system level observations made on the experimental *in vitro* systems. A way of selecting new wetlab experiments may be to give a degree of priority to those that may invalidate, or not, the current best *in silico* analogues.

1.5 Acknowledgments

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