The Energetics of Cell Sorting in Three Dimensions

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Abstract:

Dissociated aggregates of two cell types exhibit either normal sorting, partial sorting, engulfment or mixing. We show that a simple model including only differential adhesion and membrane elasticity can reproduce these behaviors. The timescale for bulk sorting in three dimensional simulations is much faster than in two dimensions and the logarithmic time dependence of two dimensional sorting is not observed.

Introduction:

An early embryo, or even an adult animal such as *hydra,* can regenerate from an aggregate of randomly mixed cells.[1-5] Even *in vivo*, during embryonic development or wound healing, cells must migrate long distances.[6] Biologists tend to think of this migration in terms of its function and the evolutionary pathways which led to it. To a physicist, the intriguing question is the mechanisms which can cause cell migration. There are only two ways that a cell can receive information from its environment, through bulk gradients in diffusible substances, or through surface properties of neighboring cells sensed by direct contact. Other information requires external fields such as light or gravity.

The former mechanism is reaction-diffusion, with analogies in chemical systems, flame fronts, and the Belousov-Zhabotinsky reaction. It may dominate cell differentiation, for example determining the sequence, or even the number, of digits in the hand.

In the latter mechanism, the sorting of cells results from cell type dependent differences in cell-cell adhesion.[8] Cells rearrange to decrease the total cell-cell contact energy. Non-specific and/or type-dependent surface adhesivity is present in cells from all multicellular organisms in the form of numerous proteins outside or across the membrane. Common adhesive molecules like N-CAM are related (possibly evolutionary precursors) to immunoglobins, and share their repeated highly variable recognition sequences.[9] Thus a cell which expresses several N-CAM like molecules can vary its adhesivity with respect to each other cell type almost independently. This mechanism allows genetics, as expressed in the choice of recognition sequences during cell differentiation to directly influence organization, perhaps, via homeotic gene pathways.

However, the physics of cell rearrangement is still unclear. The energy surface explored by the cells might have three possible textures. The first has at worst saddle points, with no local minima; so even an aggregate of passive cells could spontaneously relax to the configuration of minimum energy.[10] The second has only weak local minima. At least random cell-membrane fluctuations are necessary to allow each cell to locally explore its neighborhood and diffuse to the minimum energy configuration.[2,11] The third has deep local minima. Cells can rearrange into the optimal configuration only if they use their cytoskeletal apparatus for long range directed motion.

Experiments:

The typical experiment selects two cell types, e.g. the pigmented and neural retinal cells of chicken embryo or the endodermal and ectodermal cells of *hydra*. If necessary, one cell type is stained. The tissues are then dissociated into single cells, mechanically and/or chemically, mixed in the desired proportion, reaggregated into a random cluster, and cultured either in hanging drops, shaker flasks (chicken) or culture wells (*hydra*). The dissociation destroys any preexisting diffusible gradients. Thus, initial cell migration depends only on cell-cell surface recognition. If the dissociation procedures preserves surface adhesion molecules, cell migration begins immediately. Otherwise they must reform, and migration begins after a few hours, indicating that the main mechanism of migration is surface adhesion.

Cell sorting is the classic behavior of mixed heterotypic aggregates. In fig. 1 we show our version of a classic experiment on the sorting of neural (light) and pigmented (dark) retinal cells from seven day chicken embryos.[2,12] The initially free floating cells (a) gradually coalesce to form a random aggregate (b). During the next few hours a surface light monolayer begins to form (c) and the aggregate rounds. Even after the monolayer is complete, the dark cell clusters in the bulk remain small (d). These clusters gradually coalesce (e) until only a single dark cell cluster surrounded by light cells remains (f). The central cluster is often not centered in the aggregate.[8] Similar sorting occurs in *hydra*,[4,5] and in two-dimensional monolayers.[13]

Sometimes the sorting of cell types in an initially mixed aggregate remains incomplete. Clusters of each cell type form and grow, but never reach the final state of two connected concentric regions. Instead, the main clusters trap other large heterotypic clusters.[2,8,14] In large *hydra* cell aggregates, a monolayer of ectodermal cells forms within 6 hours but complete bulk sorting does not have time to occur.[4, 10] Partial sorting also occurs for

heterogeneous initial distributions of light and dark cells since clusters are distant and have a low probability to encounter and fuse. A more significant example occurs in chick cell aggregates in the presence of Cytochalasin B which prevents membrane ruffling,[15] showing that membrane fluctuations are necessary for complete sorting; however, detailed experiments on the amplitude of membrane fluctuations and their effect on cell sorting are lacking.

If homotypic aggregates of two types that normally sort (e.g. chicken heart and pigmented retinal cells or *hydra* endoderm and ectoderm) are brought into contact, one cell type engulfs the other yielding the same final configuration as a random aggregate.[2]

If the difference in adhesivity between the two cell types is greater than either homotypic adhesivity, spontaneous mixing occurs. For instance, during the sexual maturation of avian oviduct,[16] when the epithelial sheet of gland cells and ciliated cells forms a two-dimensional checkerboard of almost rectangular cells with minimal homotypic contact. However, the configuration retains many defects.

Simulations:

People have long tried to explain the patterns of tissues by soap froth models.[17,18] Soap froths minimize surface energy, so all we need to do to make a soap froth model into a differential adhesion model is to stabilize the cell volume as in magnetic bubbles,[19] and introduce a type dependent surface energy. There are many ways to implement this dynamics, including vertex, center, and boundary models.[14]

We use an extended large-Q Potts model[20] on a 100x100x100 next nearest neighbor square lattice,[21] with a spin, $\sigma(i,j)$, defined at each lattice site, (i,j). We assign a separate spin, $\sigma \in \{1,...,2004\}$, to each of the 2004 cells in the pattern, with all lattice sites with a given σ composing the cell σ. Each cell contains approximately 120 lattice sites.

Each cell has an associated cell type $\tau(\sigma)$. Bonds between like spins have energy 0, so the energy inside a cell is zero. Mismatched spins at cell boundaries contribute a cell type dependent surface energy $J(\tau,\tau')$. We include the cell size as a target area, A_τ, with membrane elasticity, λ. The total energy is thus:

$$H_{Sort} = \sum_{(i,j)(i',j') \text{ neighbors}} J(\tau(\sigma(i,j)), \tau(\sigma(i',j')))(1 - \delta_{\sigma(i,j),\sigma(i',j')}) +$$

$$\lambda \sum_{\text{spins } \sigma} (a(\sigma)-A_{\tau(\sigma)})^2 ,$$

where $a(\sigma)$ is the area of a cell σ. The cell types are low surface energy, **dark**, cells, high surface energy, **light**, cells and a fluid medium of unconstrained volume.

The initial condition is a random round aggregate: to obtain it, we begin with an array of rectangular cells of a single cell type and allow it to round; we then randomly reassign the cell types. We evolve using a standard Montecarlo Boltzmann algorithm at fixed temperature, allowing the nucleation of medium filled vacancies but not heterogeneous cells. One MCS is 16 times as many time steps as there are lattice sites.

The biological limit corresponds to very low temperatures.[10] The thermal membrane fluctuations are too small to be relevant. The model temperature T thus corresponds to the much bigger cytoskeletally driven membrane fluctuation amplitude. Varying it allows us to explore the energy landscape. The rate of spontaneous ($\Delta H < 0$) processes is nearly independent of T, since they have a temperature-independent probability in the Montecarlo dynamics. On the other hand, thermally activated processes depend exponentially on T. Artefacts include pinning at low temperatures and cell splitting at high temperatures.[22]

The model is simple but realistic, in that the position and diffusion of the membrane determine the dynamics as they do for real loosely aggregated cells. Relative contact energies and boundary curvatures drive all motion. Thus vertices are always close to their equilibrium condition and all topological rearrangements happen automatically and rapidly.

The model has two main defects from the perspective of biological realism. The cells are nonpolar, whereas real cells in two-dimensional tissues (epithelia) have strongly orientation dependent mechanical and adhesive properties. However, this polarity can temporarily vanish in dissociated cells. More seriously, the cell-cell adhesivity is time independent. In actual cell-cell contact, adhesion molecules diffuse into the contact region, at a rate depending on the way they are anchored in the membrane, and causing an increase in adhesion with time, which is not well understood. Long time adhesion, through adherens junctions and regeneration of the extracellular matrix are additional complications.

Results:

Normal Sorting:

We begin with a random aggregate (fig. 2(a')). Isolated cells are rapidly expelled and small clusters form within ten MCS (fig. 2(b')) and white cells rapidly begin to fill the boundary (fig. 2(b)). By 70

MCS the surface monolayer is almost complete(fig. 2(c)) and the black cells form a connected cluster (fig. 2(c')). From this point on, the sorting slows greatly. At 300 MCS only one unconnected white cell cluster remains (fig. 2 (d')) and this reconnects by 400 MCS (fig. 2 (e')). The final rounding of the dark cell cluster is very slow (fig. 2 (f')), and is not completed by 2500 MCS.

The evolution is much faster than for comparable two dimensional sorting[21,22], where the light cell monolayer requires 600 MCS, the single cluster forms at 5000 MCS, and rounding requires 13,500 MCS. Thus the ratio between monolayer formation time and sorting time is much smaller in three dimensional aggregates. Unlike for two dimensions, all lengths saturate and there is no long time logarithmic growth.

Partial Sorting:

We have not yet simulated partial sorting in three dimensions. In two dimensions,[22] we exchange J_{ll} and J_{ld}., keeping all other surface energies the same. The initial rapid clustering is nearly identical to normal cell sorting, though the dark cell clusters are smoother and rounder. However, the partial cell sorting does not form a monolayer, and even after 1000 MCS the clusters still contain many small heterotypic inclusions. The main contribution to the decrease in heterotypic contact length now comes from slow, steady cluster rounding, rather than the coalescence of diffusing clusters. Evolution is logarithmic at all times.

Engulfment:

In figure 3 we show a simulation of engulfment where we begin with our equilibrated pattern with the top half assigned to light cells and the bottom half to dark cells (fig. 3(a)). At 100 MCS the white cells have crawled around three cell diameters around the dark cell cluster (fig. 3(b)). Engulfment is complete by 700 MCS (fig. 3(c)). However, the dark cell cluster does not move to the center of the aggregate even at very long times. Engulfment is much faster than in two dimensions, where, for comparable conditions, it requires about 13,000 MCS. because a contact line is much more effective than a contact point at pulling cells, and the area to be covered decreases as the square of the distance traveled rather than linearly.[22]

Mixing:

We use experimental relative surface adhesivities to simulate the formation of a checkerboard, beginning with a random aggregate (fig. 4 (a)). Small patches of checkerboard appear immediately and grow as the two cell types rapidly intercalate. After this initial rapid reorganization, defect annealing requires long range cell motion over substantial energy barriers, and further evolution is very slow. The

surface is essentially defect free by 2100 MCS (fig. 4 (b')), but the bulk still contains long range inhomogeneities (fig. 4(b)). Timescales are similar to those observed in comparable two dimensional simulations.[22] However, unlike the two dimensional case, the growth slows faster than the logarithm. Biological checkerboards have been observed only in two-dimensional tissues.[16]

Cavities:

In two dimensions, we can simulate the nucleation, though not the stabilization, of a fluid filled hole by changing the target areas of the cell types to be unequal. In three dimensions we have not yet been able even to nucleate a cavity, because the configuration fills vacancies by relaxation too rapidly. Simulating cavity nucleation is a major topic of our current research.

Temperature and Elasticity Effects:

We have not yet studied the effects of temperature and membrane elasticity in three dimensions. In two dimensions there are three temperature regimes: low-temperature freezing, a "normal" regime which is qualitatively independent of the temperature, and a high temperature disordered regime. The existence of this broad normal band suggests that the lattice anisotropy is not too important and that we are seeing the true local minima of the pattern.

During monolayer formation, higher temperatures evolve more rapidly. These differences suggest that monolayer formation depends on thermally activated processes. However, the final state is temperature independent over a fairly wide range.

In mixing, regions of ordered checkerboard are effectively frozen at moderate temperatures. At high temperatures, cells move freely, allowing long range annealing.

Except for very low values, which do not conserve cells, migration occurs for all λ. However, λ strongly affects the relative timings of bulk and surface sorting. Bulk sorting for λ=10 is ten times slower than for λ=0.5, but monolayer formation slows by a factor of 50.

Conclusion:

Even simple mixtures of two cell types can exhibit several experimental behaviors, complete and partial cell sorting, engulfment and mixing. We wish to distinguish the behaviors explained by adhesion from behaviors which mix adhesion with other mechanical or chemical mechanisms, e.g. cytoskeletal activity, or which are not linked to adhesion at all.

Our simple model of isotropic cells, including only differential surface energies and an area constraint, can reproduce all of the

sorting behaviors of random aggregates. Thus, the energy landscape suffices to explain many aspects of biological cell sorting. Small membrane fluctuations suffice to find the optimal configuration, though the energy surface has weak local minima that trap in the absence of fluctuations.

In the simulations, the relative time dependence of the sorting depends on the fluctuation temperature and the membrane elasticity. We are currently repeating the experiments of Armstrong[15] on chicken cell sorting in the presence of Cytochalasin B, to measure comparable quantities.

The current model suggests several biological experiments. At a fundamental level, the effective temperature of cell membrane fluctuations and the nature of the cell-cell adhesion energy need to be clarified.[23,24] For example, what are the changes in cell-cell adhesivity as a function of contact time? We are trying to directly measure the cell-cell time dependent adhesivity using atomic force microscopy.

The substantial differences between two and three dimensional simulations include much faster bulk sorting in three dimensions and non-logarithmic time development. However, in our small simulated aggregate, surface cells form a substantial fraction of the total cell volume. We expect that bulk sorting will be much slower for aggregates with more cells.

We are trying to improve our Hamiltonian to provide more biological realism, adding a mesoglea, time or situation dependent surface energies and mitosis. Sine these additions take us away from the simple, physical model which motivated our initial simulations, we try to introduce as few complications as possible, and only experimentally measurable quantities.

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References:

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Figures:

Figure 1. Cell sorting between pigmented (dark) and neural (light) retinal cells in 7th day chick embryo in three dimensional aggregates. (a) Initial dissociated cells. (b) Formation of random aggregate (7 hours). (c) Monolayer formation (8 hours). (d) Rounding of aggregate, completion of surface layer (15 hours). (e) Bulk sorting (60 hours). (f) Final sorted state (>60 hours). Pictures show top views of several different three dimensional aggregates.

Figure 2. Simulation of Three Dimensional Cell-sorting. (a, a') Initial configuration: random aggregate. (b, b') 10 MCS. (c, c') 70 MCS. (d, d') 300 MCS. (e, e') 400 MCS. (f, f') 2500 MCS. Parameters: J_{ll}=7, J_{dd}=2, J_{ld}=5, J_{lM}=J_{dM}=8, T=32, λ=1.

Figure 3. Simulation of Three Dimensional Engulfment. (a, a') Initial configuration. (b, b') 100 MCS. (c, c') 700 MCS. Parameters: J_{ll}=7, J_{dd}=2, J_{ld}=5, J_{lM}=J_{dM}=8, T=32, λ=1.

Figure 4. Simulation of Three Dimensional Checkerboard. random initial cell type assignment. (a, a') Initial configuration. (b, b') 2100 MCS. Parameters: J_{ll}=6, J_{dd}=4, J_{ld}=2, J_{lM} = J_{dM} = 8, T=32, λ=1.