# Do Endothelial Cells Dream of Eclectic Shape?

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Endothelial cells (ECs) exhibit dramatic plasticity of form at the single- and collective-cell level during new vessel growth, adult vascular homeostasis, and pathology. Understanding how, when, and why individual ECs coordinate decisions to change shape, in relation to the myriad of dynamic environmental signals, is key to understanding normal and pathological blood vessel behavior. However, this is a complex spatial and temporal problem. In this review we show that the multidisciplinary field of Adaptive Systems offers a refreshing perspective, common biological language, and straightforward toolkit that cell biologists can use to untangle the complexity of dynamic, morphogenetic systems.

## Introduction

The variety of forms and contortions that endothelial cells (ECs) exhibit and collectively coordinate during vascular development, adult homeostasis, and pathology is truly staggering (Figure 1). EC shape is incredibly eclectic, which means that unlike red blood cells, with their relatively fixed forms, there are a wide variety of possible EC shapes. Moreover, EC shapes are determined adaptively—organisms have evolved to generate dynamic EC forms such that they optimize the collective, vascular network structure and function in response to, and generating feedback with, the larger tissue environment. To actively adapt their form to environmental changes, single ECs integrate multiple signals and coordinate collectively to allocate transient shapes/behaviors to certain individuals, while at the same time balancing the pressure and forces from blood flow and external tissue environment along the whole vessel (Geudens and Gerhardt, 2011; Humphrey, 2008).

To perform this adaptive, complex morphogenetic feat, individual cells need sensory systems to detect external signals, an internal control/decision infrastructure to integrate signals with the cell's current prevailing state, and structural machinery to change the cell's shape/behavior accordingly. En masse, dynamic feedback between these key components in each cell, as well as with the cell's immediate physical and chemical environment, leads to collective self-organization of structure, generating complex network-level phenomena over time.

In the previous paragraph, we introduced the Adaptive Systems (AS) conceptual framework in a vascular setting. Notably, this may have gone undetected, as it uses the same language as experimental biology. This is perhaps the first and important triumph of the AS field, namely that it does not have to transcend disciplines and language barriers. AS was developed from the start as a multidisciplinary effort by biologists, computer scientists, engineers, and roboticists with a common goal and common language: to understand the underlying principles of adaptive systems, be they biological, simulated, or instantiated in robots. Mathematical tools have previously been identified as highly illuminating and important for understanding biological complexity (Kitano, 2002; Lewis, 2008; Tomlin and Axelrod, 2007). However, due to communication roadblocks and cultural misconceptions, a gulf still remains between mathematics and many areas of biological experimentation (Di Ventura et al., 2006). The AS framework can provide a refreshing escape from this cultural gap, allowing us to directly tackle the mechanisms underlying single to collective cellular behavior with disciplinary synergy. Thus, here we introduce the AS perspective as it relates to vascular biology and the wider biological domain. For the reader interested in the computational methods used to program and build AS models, we refer you to Bonabeau et al. (1999), Camazine (2003), and Grimm and Railsback (2013). Here we will focus instead on how experimentalists can interact with, and benefit from, the AS approach for their research. We therefore provide a straightforward guide, using plain English (and no equations!), to integrating simulated ("simulant") cells with experimentation in order to generate new experimentally relevant data, as well as mechanistic insight into the temporal and spatial feedback of morphogenesis.

Drawing parallels with the evocative robotic humans (androids) in Philip K. Dick's novel *Do Androids Dream of Electric Sheep?*, which inspired the masterful film *Bladerunner* by Ridley Scott, whose behavior serves as a mirror to view, question, and understand our own behavior, we posit that we can learn about real cells by watching the interaction of individual simulant cells in the "virtual lab" as they collectively generate new and unexpected tissue-level dynamics. Taking the *Bladerunner* analogy further, we can aspire to study "rogue simulant cells." Rogue simulants, instantiated with mutations and/or let loose within untested pathological environments, can produce unexpected aberrant behavior. This can provide novel insight into maladapted behavior in real cells and uncover new avenues to explore for therapeutics.

## Endothelial Cells: You Are All Individuals!

At the heart of an AS approach to understanding morphogenetic systems such as the vasculature is to explicitly characterize the vessels as composed of autonomous, individual entities (single ECs). The properties of adaptive individuals, whether they are cells, animals, robots, or simulant cells, can then be broadly decomposed into the following functional subclasses: sensors (to sense the environment), controller (to generate a decisive outcome), and effectors (to implement behavior and alter shape and position) (Figures 2A–2D). These can overlap, in that a

physical component or molecular complex can be multifunctional—both a sensor and an effector—adhesions, for example, which contribute to force and traction, but also act as receptors (Giannotta et al., 2013). However, considering the functionality separately (e.g., sensor and effector) can reveal feedback dynamics, which are otherwise hard to intuit.

When we consider the vasculature, this decomposition appears trivial:

(1) Sensors: ECs have receptors, mechanotransductors, and even electrical transducers to sense the environment (Huang et al., 2013; Ross et al., 2013; Simons, 2004);

(2) Controller: signal transduction pathways and genetic regulatory networks integrate multiple signals to "decide" on responses (Regan and Aird, 2012; Friedl and Wolf, 2010; Vitorino and Meyer, 2008); and

(3) Effectors: the cytoskeleton and adhesions generate shape change and movement (Fraccaroli et al., 2012; Giannotta et al., 2013; Ingber et al., 1995).

However, individual cell-level autonomy in determining each cell's own time-variable behavior is not often considered in everyday experimentation, where many techniques provide averaged, static, population-level data. For example, western blot analysis and quantitative PCR measure average protein and mRNA expression, respectively, across millions of cells. The signaling insights are then attributed to individual single cells as if all cells behave the same. An average expression level may indeed indicate that all cells react equally to the stimulus, but it can also mask their heterogeneity, namely that some cells have much higher and others much lower levels than average. Heterogeneous versus homogeneous behavior of individuals can drive markedly different tissue-level dynamics (Bentley et al., 2009; Huang, 2009; Tambe et al., 2011). Thus, one of the major benefits of thinking about multicellular systems from the perspective of individual, autonomous cell behavior is that it allows us to explicitly consider such heterogeneity. Live imaging approaches that can capture single EC shape dynamics are therefore highly synergistic with the overall AS approach. For example, live imaging of in vitro chimeric embryonic stem cell sprouting assays and the in vivo zebrafish embryo revealed the single-cell rearrangement behaviors contributing to vessel sprouting and anastomosis (Blum et al., 2008; Jakobsson et al., 2010). Increasingly, such single-cell resolution experimental models will be able to inform, and be informed by, AS simulant models of single to collective cell morphogenesis.

### Controllers: How Do Individuals Decide to Change Shape?

How do autonomous individuals integrate the myriad of external signals into concerted actions or responses? In robotic adaptive systems, the design of an adaptive controller is a central problem. A controller that can select the most appropriate action for a robot to perform at a given time, acting in the real world in response to the environment around it ("action selection"), initially proved nearly intractable. This was due to the use of traditional artificial intelligence (AI) approaches, which exhaustively considered all options and planned out each response in turn, to find the best one (Maes, 1993). Later controller designs, which exploited biological controller properties such as bistable

feedback and modularity, paved the way for a new era in AI, with "bioinspired robotics" exhibiting and providing a test-bed to investigate a wide variety of adaptive behavior in a faster, cheaper manner (Brooks, 1986; Pfeifer et al., 2007).

This bio-inspired design approach revealed that the organization of the controllers' constituent parts, not just the parts themselves, is key to generating the adaptive behavior. When environmental cues change past certain thresholds, cells can abruptly change their behavior. This behavioral change is typically mediated by "bistable" regulatory feedback mechanisms, including circuits with positive or double-negative feedback or epigenetic regulation (Brandman and Meyer, 2008; Regan and Aird, 2012; Tyson et al., 2003). Bistability requires the existence of a barrier between two states, such that transient signals can push a cell from one of its stable states into the other. This endows cells with memory: its current state, which was affected by its previous state, will affect the choice of its next behavior. This means that the autonomous generation of behavior in an individual can greatly differ from the response of its neighbor cells to the same stimuli, if their internal states are currently different (Figure 2C). For example, ECs exposed to inflammatory stimuli may become activated or maintain their noninflammatory phenotype, depending on the level of shear flow they have been experiencing (Tsai et al., 2007).

An intriguing interdisciplinary study of EC sheet migration demonstrated the role of modularity in controllers. Vitorino and Meyer (2008) used a large RNAi screen to knock down 2,400 signaling molecules and found that the failure mode of sheet migration, when present, was modular in nature. The breakdown of the overall phenotype was nearly always restricted to one of four aspects of sheet migration—cell motility, directed migration, cell-cell coordination, or cell density—while the other three were left untouched. The authors showed this to hold true dynamically using simulant cells with modular controllers (Vitorino et al., 2011) and concluded that there are separate regulatory modules responsible for proliferation, migration speed, chemotaxis, and coordination among neighbors (Figure 2C). Exploration of modular behavior switching has also recently been performed using simulant cells in different environments with results compared against in vitro sprouting assay data (Long et al., 2013).

### Individuals Are Not Separable from Their Body or Their Environment

The controller with its internal regulators and switches is, however, not the sole or necessarily key determinant of many adaptive behaviors, though it tends to attract the most credit. Individual ECs are (1) situated, in that they have an environment with which they interact with two-way feedback, and (2) most importantly, they are embodied: the cells have a structure (morphology), which constrains and modifies their actions (Brooks, 1990, 1991; Maes, 1993; Pfeifer, 1996; Pfeifer et al., 2007). Explicitly considering the role of an individual's embodiment is the AS framework's prime asset, the central tenet that sets it apart from other approaches, and can lead to the identification of often overlooked, simple feedback mechanisms underlying morphogenesis and "complex" behavior.

These concepts are perfectly illustrated by Braitenberg's Vehicles (Braitenberg, 1986), in which complex adaptive
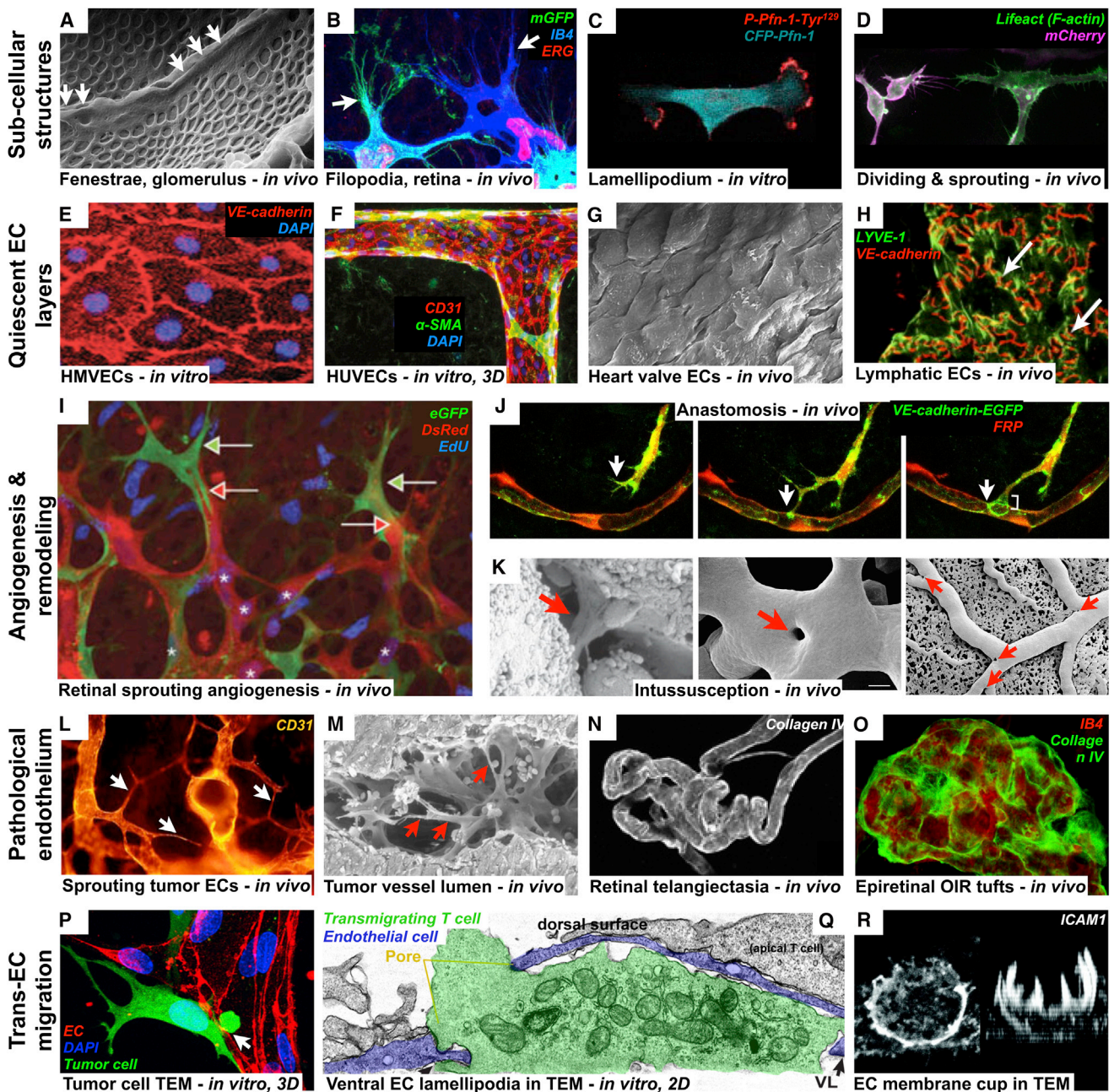
**Figure 1. Endothelial Cells Display a Startling Repertoire of Shapes**

(A) Fenestrae and tight junctions: lumenal side of a glomerular capillary showing two adjacent ECs with striking arrays of round fenestrae that cover their entire surface. The raised ridge (arrows) represents the tight junction between the two cells. Reprinted from Rice et al. (2013).

(B) Filopodia: confocal images of chimeric retinas, derived by injection of SRFfl/wt/PDGFb-iCreER/mTmG ESCs into wild-type embryos. White/green arrows indicate host-derived (wild-type)/SRFfl/wt/PDGFb-iCreER/mTmG ESC-derived filopodia bursts (blue, isolectin B4; red, Erg). Reproduced with permission from *Development* (Franco et al., 2013) (http://dev.biologists.org/content/140/11/2321).

(C) Lamellipodium: migrating human microvascular ECs transfected with pECFP-Pfn-1 (cyan), treated with anti-P-Pfn-1-Tyr129 antibody (red). Reprinted by permission from Macmillan Publishers Ltd: *Nature Cell Biology* (Fan et al., 2012), copyright 2012.

(D) Division and sprouting: daughter ECs with rounded cell bodies (left) near an intersomitic vessel (right). Membrane dynamics within one daughter EC are nonuniform, with polarized filopodia on the right and smooth membrane on the left (green, LIfeact F-actin probe; magenta, mCherry-labeled membrane). Courtesy of Li-Kun Phng.

(E) Confluent EC monolayer: confluent monolayer of human microvascular endothelial cells in a microfluidic channel in vitro (red, VE-cadherin; blue, DAPI). Reprinted by permission from Macmillan Publishers Ltd: *Nature Protocols* (Huh et al., 2013), copyright 2013.

(F) Cobblestone ECs in a microvessel: endothelial-pericyte interactions in an in vitro microvessel network (red, CD31; green, α-smooth muscle actin; blue, nuclear DAPI). Reprinted by permission from Macmillan Publishers Ltd: *Nature Protocols* (Morgan et al., 2013), copyright 2013.

(G) Cobblestone ECs in a heart valve: scanning electron photomicrograph of ECs lining a porcine heart valve. Reprinted from *Biomaterials* (Brody et al., 2007), with permission from Elsevier.

behavior emerges from the interactions between the vehicles' very simple embodiment (sensor and motor placement/connections) and environment in the absence of a controller. The most well-known vehicle is illustrated in Figure 2E (part 1). It has just two light sensors at the front and two wheels at the rear. The left-hand light sensor input is directly linked to drive the right-hand wheels' speed (effector of motion) and vice versa. This crossover sensor-effector morphological structure alone is sufficient to generate robust phototaxis behavior by exploiting feedback with the environment. The individual does not calculate the location or even direction of the light, nor does it "know" when it should stop turning. Rather, it adaptively behaves by simply exploiting dynamic feedback between the environment and its sensors/motors; a stronger light input to the left will drive the right-hand wheel, which turns the vehicle left toward the source (Figure 2E, part 1). While Braitenberg's work was a thought experiment, even the simplest real world robotic versions show further unexpected, novel behavior: slowing down in a "reflective" fashion as they encounter dark areas and exhibiting obstacle avoidance through avoiding the shadows of objects (Figure 2E, part 2). This concept of "emergent," novel behavior in such robots, or more abstractly, in any situated and embodied system, was perhaps best characterized by Brooks: "The intelligence [adaptive behavior] of the system emerges from the system's interactions with the world and from sometimes indirect interactions between its components—it is sometimes hard to point to one event or place within the system and say that is why some external action was manifested" (Brooks, 1991).

An individual's behavior is shaped by the spatial organization and interface of its body with the environment. The unique sensory "umwelt" of each individual cell can drive very different behavior from a seemingly similar cell nearby (Von Uexküll, 1920) (Figure 2B). For example, if we simply switch the position of the light sensors on Braitenberg's vehicle, we find that its behavior has flipped; rather than phototaxis, the vehicle now runs away from light and appears to "hide" in the darkest corner

of the room (Figure 2E, part 3). An intriguingly similar study highlighted the importance of structural interconnections between sensors and motors to overall organism behavior in vivo. Kullander et al. (2001) modified the strict contralateral connections of motor neurons in mice, which normally cross at the midline such that one side of the brain controls motion for the opposing side of the body. They developed mutants where the axons instead freely crossed back and forth in the motor cortex, generating bilateral motor control. The mutant mice then exhibited a curious hopping gait rather than the alternative stepping of normal mice (Kullander et al., 2001). Further, disrupting the *location* of a sensor, and not its expression level or its connections, can have equally drastic effects on cell behavior. Such a phenomenon has been observed in silico when varying lumenal versus ablumenal localization of VEGFR-1 and VEGFR-2 on blood vessels, which resulted in surprisingly high changes to the distribution of VEGF in tissue or in blood (Stefanini et al., 2009). Equally, experimental disruptions of polarity, such as apical/basal polarity in ECs, which determines the localization of specific molecules to lumenal/ablumenal surfaces, showed changes in cell shape, from flat to cuboid in vitro (Lizama and Zovein, 2013; Zovein et al., 2010) and to the entire lumenal collapse in sprouting vessels (Lampugnani et al., 2010).

The precise number of receptors on different ECs and how they vary over time has recently begun to be revealed using an integrated bioengineering approach (Imoukhuede et al., 2013; Imoukhuede and Popel, 2011), and simulations have been used to understand the effects on sensing of the dynamic interplay and heterodimerization of multiple receptors on the surface (Mac Gabhann and Popel, 2004, 2007). Indeed, receptors move over time, within membranes or by diffusing into extracellular space, further extending and altering each cell's umwelt. Soluble VEGFR-1 (s-flt1) is released by ECs and acts as a sink, locally depleting VEGF signals for neighbor cells to sense as new vessels sprout in angiogenesis (Chappell et al., 2009). A recent integrated in silico/in vitro study identified

(H) Button junctions in lymphatic vessels: dexamethasone-induced button formation (arrows) between oak leaf-shaped lymphatic ECs in neonatal mouse trachea (red, VE-cadherin; green, LYVE-1). Reprinted from *The American Journal of Pathology* (Yao et al., 2012), with permission from Elsevier.
(I) Sprouting angiogenesis: mosaic vascular front in retina of a mouse derived from eGFP-expressing blastocyst (greed) injected with DsRed-expressing embryonic stem cells (red), stained for EdU (blue) to mark proliferating ECs. Reprinted by permission from Macmillan Publishers Ltd: *Nature Cell Biology* (Jakobsson et al., 2010), copyright 2010.
(J) Anastomosis: time-lapse images of anastomosis between the communicating vessel that links the prosencephalic artery to the center of the palatocerebral artery (PLA) in the developing zebrafish head. The leading tip cell (left, arrow) connects to an EC body within the PLA, making a spot of junctions (middle, arrow). The newly formed ring connects to an existing junction line on the PLA (right, arrow) and transcellular lumen forms in the tip cell, upward from the PLA (right, white bar; green, VE-cadherin; red, FFP). Reprinted from *Developmental Cell* (Lenard et al., 2013), with permission from Elsevier.
(K) Endothelial intussusception. Left: endovascular pillars (arrow), the corresponding intralumenal structures to the pores of intussusception (MxCre Notch1lox/lox mice), are shown. Reprinted from *Gastroenterology* (Dill et al., 2012), with permission from Elsevier. Middle: tissue pillar (arrow) extending across the capillary lumen (scanning electron micrograph of a Mercox microvascular cast, rat lung at P4; bar, 3 $\mu$m). Right: intussusception in branching remodeling of the chick chorioallantoic membrane (vascular cast; embryonic day 8–10). Four of five visible bifurcations embrace a transcapillary pillar (arrow: heads/holes in cast). Reprinted from *Molecular Aspects of Medicine* (Burri and Djonov, 2002), with permission from Elsevier.
(L) Hypersprouting in tumor vessels: tiny (1 $\mu$m diameter), lumen-free sprouts (arrows) interconnect tumor vessels (MCa-IV tumors; orange, CD31 immunoreactivity by Cy3 fluorescence). Reprinted from *The American Journal of Pathology* (Hashizume et al., 2000), with permission from Elsevier.
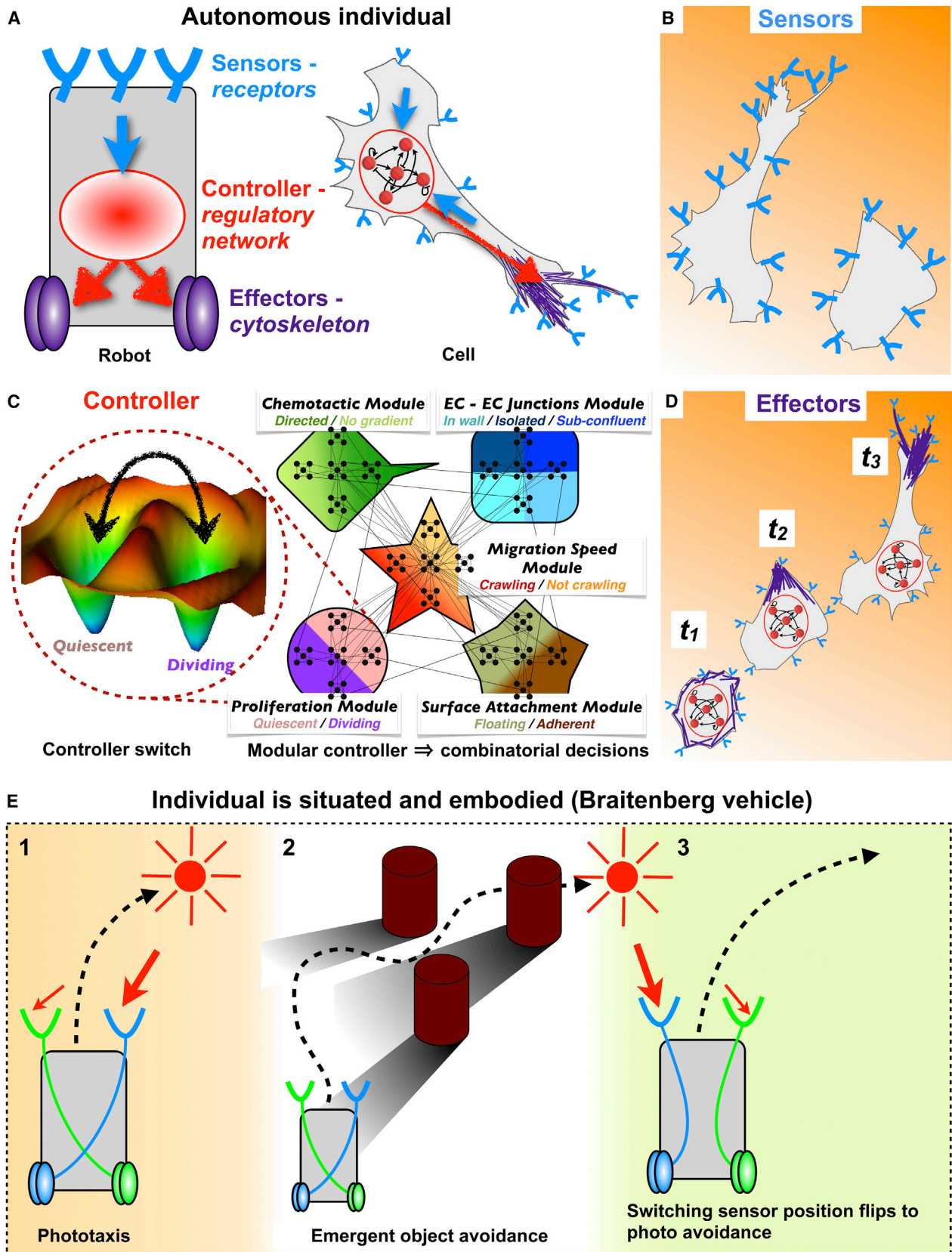(M) Intralumenal abnormalities in tumor vessels: lumenal surface of the tumor vasculature, where ECs with abnormal shapes partition the lumen (arrows; scanning EM of MCa-IV tumors, mammary gland). Reprinted from *The American Journal of Pathology* (Hashizume et al., 2000), with permission from Elsevier.
(N) Corkscrew-like retinal capillaries: vascular remodeling in the retinal of dystrophic RCS rats (20 weeks; white, collagen IV). Reprinted from *The American Journal of Pathology* (McKenzie et al., 2012), with permission from Elsevier.
(O) Epiretinal tufts in oxygen-induced retinopathy (red, isolectin; green, collagen IV). Reprinted from Håkansson et al. (2011).
(P) Transendothelial migration (TEM): tumor cell in mid-TEM, crossing the wall of an in vitro microvessel. Reproduced from Chen et al. (2013) with permission of The Royal Society of Chemistry (http://dx.doi.org/10.1039/c3ib40149a).
(Q) Ventral lamellipodia (VL) aid closure following T cell transendothelial migration. Ultrastructural view of basal F-actin-rich protrusions putatively representing VL (arrows) during late stages of T cell (green) diapedesis across the endothelium (blue). Courtesy of Christopher V. Carman; originally published in *Journal of Cell Biology*, http://dx.doi.org/10.1083/jcb.201209077 (Martinelli et al., 2013). Cup-shaped microvilli-like membrane projections enriched in ICAM-1 in HUVECs bound to activated T cells (white, ICAM-1; left/right, top/side view projections). Copyright 2003, *The American Association of Immunologists, Inc.* (Carman et al., 2003).

**A** Autonomous individual

Sensors - *receptors*

Controller - *regulatory network*

Effectors - *cytoskeleton*

Robot    Cell

**B** Sensors

**C** Controller

*Quiescent*    *Dividing*

Controller switch

Chemotactic Module
*Directed / No gradient*

EC - EC Junctions Module
*In wall / Isolated / Sub-confluent*

Migration Speed Module
*Crawling / Not crawling*

Proliferation Module
*Quiescent / Dividing*

Surface Attachment Module
*Floating / Adherent*

Modular controller ⇒ combinatorial decisions

**D** Effectors

$t_1$    $t_2$    $t_3$

**E** Individual is situated and embodied (Braitenberg vehicle)

1    2    3

Phototaxis    Emergent object avoidance    Switching sensor position flips to photo avoidance

*(legend on next page)*

that this process could ultimately affect network branch spacing (Hashambhoy et al., 2011).

## Sensorimotor Feedback: Active Perception of the Environment

In early work, the philosopher Dewey elegantly characterized a crucial feedback that arises in embodied individuals: "We begin not with a sensory stimulus, but with a sensorimotor coordination … In a certain sense it is the movement which is primary, and the sensation which is secondary, the movement of the body, head, and eye muscles determining the quality of what is experienced" (Dewey, 1896). Consider how you approach a jigsaw puzzle: without feedback between movement and sensing, it simply would not be possible (Figure 3A). Von Uexküll's early cybernetics research into sensorimotor feedback (Von Uexküll, 1920) has since become established as fundamental in early childhood development (Piaget and Inhelder, 1967) and for understanding vision in neuroscience and robotics (Pfeifer, 1997).

Sensorimotor feedback is inherent in both real and embodied simulant cells. For example, during tip cell migration, cytoskeletal effectors are stimulated by VEGF receptor activation to generate long, thin protrusions of the cell's membrane (filopodia), which relocates the cell's receptors, bound to the cell's deforming membrane, further into VEGF gradients. This in turn triggers greater stimulation. Thus, the shape changes alter the interface between the cell and its environment along which the next iteration of feedback is generated, in this case providing positive feedback to the stimulatory pathways and speeding up migration in steeper gradients (Bentley et al., 2008; Szabo et al., 2007). Tip cell migration has been widely shown to be heterogeneous during angiogenesis due to Notch regulation of VEGFR expression levels (Geudens and Gerhardt, 2011; Hellström et al., 2007). Cells with higher levels of VEGFR activation upregulate Dll4 ligands, which bind to Notch receptors on neighboring cells, inhibiting the neighbor cells' migratory responses. A simulant study of the positive sensorimotor feedback of filopodia extension, combined with the negative feedback of Notch regulatory control, predicted an unexpected, temporal determinant of vascular branch spacing (Bentley et al., 2008). If the sensorimotor feedback is weakened (due to either a shallow VEGF gradient or loss of actin protrusion ability), the collective simulant "decisions" to select tip cells (via Notch) were slower, which corresponded to hypo- or hyperbranching depending on the relative strength of Notch signaling (Figures 3B and 3C).

The parable of Simon's ant further exemplifies this concept of environment-system feedback cycles being at the root of complex, adaptive behaviors (Figure 3D). Consider the path of an ant walking along a beach, avoiding obstacles but, overall, heading to a goal: "An ant, viewed as a behaving system, is quite simple. The apparent complexity of its behavior over time is largely a reflection of the complexity of the environment in which it finds itself" (Simon, 1996). For cells, the feedback between environment and their morphology is crucial and can even determine the choice to live or die, without the need for genetic mutations (a change in the controller) (Chen et al., 1997; Mammoto and Ingber, 2009; Werfel et al., 2013). Indeed, a recent study using a situated and embodied simulant cell, which could locally alter its shape in relation to its current physical umwelt, identified environmental determinants as key drivers of cancer cell migration decisions, which was then confirmed in vivo (Tozluoğlu et al., 2013).

## From Situated/Embodied Individuals to Collective Adaptive Patterning

The whole is greater than the sum of its parts. Collective robotics investigates the multiscale aspects of adaptive morphogenesis and behavior in a "bottom-up" manner by exploiting emergent feedback between the autonomous individual robot's component parts. Collective aggregate robots are able to adapt their form to perform different tasks that prove too complex for a single robot alone (Dorigo et al., 2012; O'Grady et al., 2010). The benefits of exploring multiscale cellular dynamics using collective robotics have recently begun to be discussed (Rubenstein et al., 2009). The recent "kilobot" project, so named as the collective can number a staggering 1,000 (Rubenstein et al., 2012), is specifically designed to investigate, in an embodied, situated fashion, how collective aggregates self-organize to generate higher-level shapes and structures (Figure 3E).

Simulation is, however, currently more amenable for cell biology than robotics is. Individual-based simulations, often called "agent-based models" (ABMs), are the natural extension of the AS perspective. Individuals or "agents" are embodied (attributed with sensors, controllers, and effectors) and are placed at different positions within a defined (situated) environment (Figure 4A). Individuals are initialized with their various component parts in a set of states, which can then dynamically change over time as the individuals interact with one another and their surroundings. Emergence/self-organization of higher-level network structures in vasculogenesis has been well captured by simulations of multiple simulant cells altering their shape and behavior in a coordinated manner, predicting, for example, preferential migration along elongated cells (Figure 3F) (Merks et al., 2006; Szabo et al., 2007). Recently a high level of dynamic, competitive cell rearrangement has been observed within collective blood vessel sprouting (Figure 3G)

**Figure 2. Adaptive Systems Framework for ECs**
(A) Autonomous individuals. For autonomous adaptive behavior to emerge, robots, simulants, and cells alike must be equipped with sensors (blue), a controller (red), and effectors (purple).
(B) Sensors are located spatially on the individual's morphology, determining their unique "umwelt": the environment as perceived by their sensors. Note the difference in perceived environmental gradient (orange) between the two cells, leading to differences in behavior between neighbors.
(C) Controller. Bistable switches confer memory and stability to cell behavior, while a modular architecture allows cells to multitask and confers robustness to modules against breakdown in other modules.
(D) Effectors. The cytoskeleton is the major effector of behavior in morphogenetic systems, as it generates structure, movement, and morphological plasticity.
(E) Situatedness and embodiment illustrated by Braitenberg vehicles. (1) The sensor on the right receives more light, is directly wired to the left-hand motor, and turns the vehicle toward the light. The resulting phototaxis is adaptive, always correcting the motion to keep moving toward the light. (2) When instantiated in real robots, Braitenberg vehicles displayed the unexpected behavior of perfect obstacle avoidance. Their simple sensor-motor coupling design turns them away from obstacle-generated shadows. (3) A simple switch of sensor positions results in light avoidance. In this case, when the left sensor detects more light, it drives the left wheel, turning the vehicle to the right and away from the light.
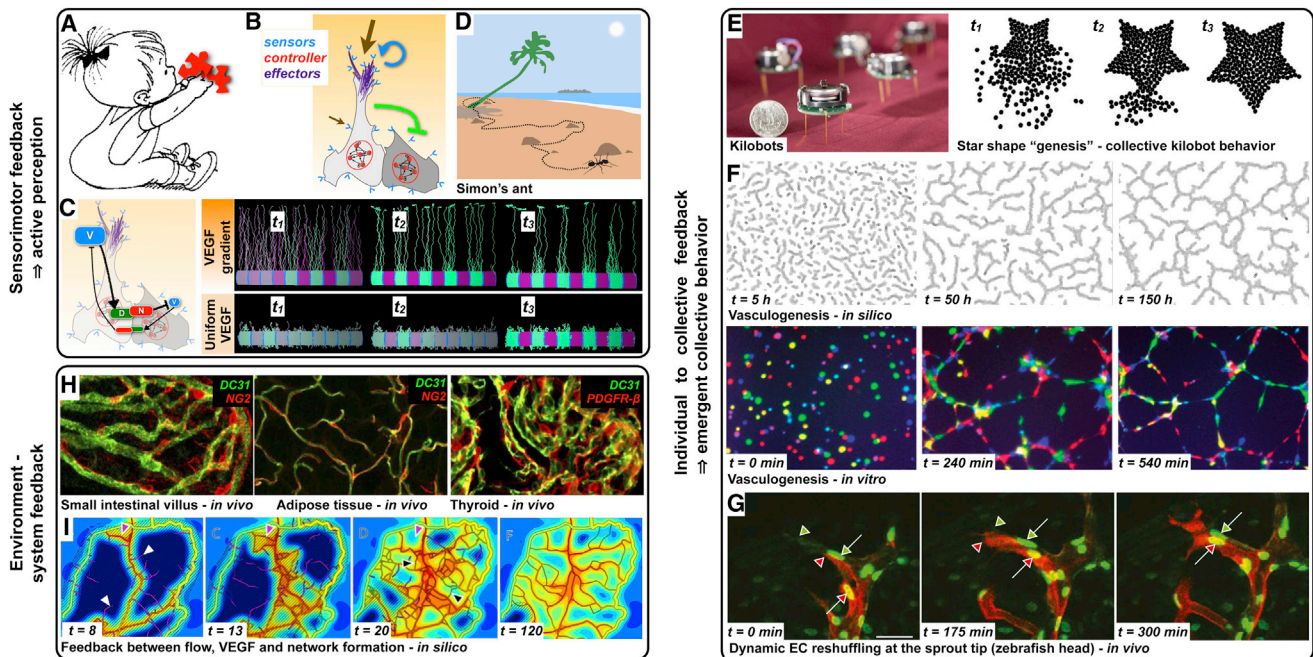
**Figure 3. Emergent Feedback in Adaptive Systems**

(A–D) Sensorimotor feedback is indispensable for many tasks. (A) Consider solving a jigsaw puzzle without the ability to move the pieces around and actively perceive how they fit together. (B) Sensorimotor feedback in ECs: as the cytoskeleton pushes the cell membrane, and therefore also the ECs' receptors, into regions with higher VEGF levels, the cell increases its own sensory input (positive feedback). This ultimately results in stronger inhibition via Notch signaling. (C) Schematic showing details of the feedback loop in (B). VEGF-VEGFR binding increases activity of the VEGFR-2 receptor (pVEGFR-2 levels, indicated by "V"), which leads to the upregulation of Dll4 ("D") ligands, which in turn bind to Notch1 ("N") on a neighbor EC, altering expression of the VEGFRs and reducing the overall levels of p-VEGFR-2 on that cell. Simulant ECs in different VEGF environments predict that in the absence of a gradient, cells lack sensorimotor feedback, and thus lateral inhibition is slower and branching is disrupted. Reprinted from *The Journal of Theoretical Biology* (Bentley et al., 2008), with permission from Elsevier. (D) The parable of Simon's ant. Complex adaptive behavior of the ant is observed globally, but it is generated by simple local rules of interaction of the ant with its complex immediate surroundings (Simon, 1996).

(E) Kilobots aid collective pattern formation studies. Left: reproduced with permission from the Self-Organizing Systems Research Group at Harvard University. Right: copyright 2010 IEEE. Reprinted with permission from Rubenstein and Shen (2010).

(F) Top: vasculogenesis, in silico. Reprinted from *Current Topics in Developmental Biology* (Czirok et al., 2008), with permission from Elsevier. Bottom: vasculogenesis, in vitro. Reprinted with permission from Parsa et al. (2011).

(G) Dynamic, collective EC position rearrangement during angiogenic sprouting in vivo. Reprinted by permission from Macmillan Publishers Ltd: *Nature Cell Biology* (Jakobsson et al., 2010), copyright 2010.

(H) Organ-specific heterogeneity of the microvessel network. Microvessels in small intestine (left), adipose (middle), and thyroid (right) tissue (green, CD31; red, NG2/PDGFR-β). Reprinted with permission from Kamba et al. (2006).

(I) Feedback between flow and environmental signals. In silico model of feedback between oxygen, VEGF, network formation, and remodeling. Reprinted from Secomb et al. (2013).

(Arima et al., 2011; Jakobsson et al., 2010) and previously in vasculogenesis (Perryn et al., 2008). Comparison of homogeneously or heterogeneously moving simulant cells in a vessel predicted that differential Notch signaling is a driving factor of the rearrangement (Jakobsson et al., 2010). This model was recently extended to include junctional adhesion and cortex movements, as effectors of rearrangement motion in the simulant cells. The model led to the novel prediction that Notch inhibition of VE-cadherin-mediated adhesion and junctional cortex dynamics contributed to the generation of heterogeneous, functional overtaking among ECs. This simulant prediction was then confirmed by extensive in vitro and in vivo experimentation (Bentley et al., 2014). For a thorough, excellent review of the different computational models addressing endothelial rearrangement, see Czirok (2013). Indeed, the benefit of simulation to capture and investigate collective, multiscale dynamics of ECs is a theme of many existing review papers, for example Qutub et al. (2009) and Walpole et al. (2013). We also refer the interested reader to an

excellent review of the subcellular-, cellular-, and tissue-level mechanical vascular adaptations (Humphrey, 2008).

Vascular networks exhibit diverse form and function in different organs and tissue environments (Figure 3H) (Aird, 2007). Feasible computational runtimes have thus far constrained the level of single-cell autonomy and shape change when simulating whole vascular network-level dynamics. However, good representation of the local tissue-vessel feedback in terms of oxygenation dynamics and/or mechanics can give new insights and startlingly realistic vascular remodeling (Figure 3I) (Bartha and Rieger, 2006; Pries et al., 2009; Szczerba et al., 2009; Zakrzewicz et al., 2002; Watson et al., 2012). The field is now moving, with increases in computational power, toward greater cellular resolution and autonomy in the generation of multiscale network-tissue-level structures (Carlier et al., 2012; Harris et al., 2013). For example, a recent hybrid model considered both force balance and detailed individual cell-level signaling and behaviors, e.g., via TGF-β, in artery/tissue

dynamics during hypertension (Thorne et al., 2011), an approach that holds promise for future integrated study of the complexity of cellular adaptive behaviors coupled to environmental feedback.

### Using Simulant Cells to Gain Novel, Experimentally Relevant Data

The big challenge ahead is no longer "can we build simulations of cellular systems?" This has been achieved time and time again. The challenge is: can we gain novel, experimentally relevant insight with simulations that will generate new experiments and data? Simulations are often wrongly perceived as simply "decoration" or as only capable of confirming a known biological phenomenon. This limited view of their worth is in part to due to (1) the lack of experimentally driven questions asked of the simulants when built by modelers in isolation from biological data; (2) a lack of clarity among modelers and experimentalists alike on what tools and approaches can be implemented to gain new experimentally relevant data; (3) communication barriers, linguistic and cultural, to disseminating ideas and insights from the simulants to the experimental biology community; and (4) oversimplified or missing AS components in the simulant's design, which restrict emergent feedback and thus limit their predictive capabilities.

We aim here to begin a real dialog, as an integrated cell biology community, to begin to address these challenges. Simulants built with care, which explicitly define the components we have discussed, will be capable of generating unforeseen novel behavior through emergent feedback. They can directly lead to new experiments and new ways of thinking, generating high-impact data and understanding. We seem to be only at the brink of realizing this potential in cell biology research. However, simulation is integrated and capitalized upon with huge effect in many other fields—for example, physics, astronomy, chemistry, sociology, economics, and epidemiology. We refer the reader to a fuller discussion of the ability of simulation to drive new and useful data and indeed reap Nobel prizes in Bentley et al. (2013).

### *Symbiotically Build, Test, and Refine Simulants with Experimentation*

The process of formalizing and defining a step-by-step, complete working model of a biological system, by which a certain behavior may occur, can be incredibly informative even before simulation takes place. We may think we understand a biological process, but it's not until we try to build a working version, explicitly defining each aspect of the system in turn, from the individual players—sensors, controllers, effectors—and considering each cell's spatial umwelt, that we realize new and missing links. This inevitably leads to a wealth of new and well-directed experiments, which are more likely to be carried out if experimentalists are part of the simulant's design committee (Kouklis et al., 2003; Morelli et al., 2012). If a roadblock to integration is that simulations have not provided enough new data on the important questions of the experimental field, then the solution surely is for experimentalists with their "hands on the pulse" to get involved and direct them there. If there is an unknown burning question that is too hard to test currently in vivo, it may be perfect for simulants to test out at a fraction of the cost, with the added benefit of providing an alternative to excessive animal testing, stated as a mandatory consideration by most Institutional Animal Care and Use Committees (IACUC).

The only way to know if your simulant is behaving correctly is, of course, to test it. Simulants should ideally be tested against multiple experimental data sets, including matching the relevant known mutant phenotypes, to check that all components are working as they should. Be wary of a model that matches to only one data set or is validated by "having a realistic-looking network shape." Going back and forth between in vivo/in vitro and in silico methods, with open communication throughout, will maximize both the realistic adaptive capacity of the simulant cells and stimulate new experimental ideas. This "symbiotic approach" (Figure 4B) is described more fully in Bentley et al. (2013).

### *Effectors that Generate Behaviors Are as Important as Their Regulators*

When designing a simulant cell system, one must include the key components discussed above; otherwise, the study may carry the "ABM" name but will not be able to generate the different levels of feedback between components, morphology, and environment in the same way as the biological system. Often overlooked or oversimplified in simulations is embodiment, in particular effectors and adaptive cell morphology. As characterized by Webb, robot building never suffers from simplification of this component: "Unlike simulation, a robot model cannot choose an arbitrary form of input to avoid the sensing problem, or have an interpreted output that skips the actuator problem. The behavior has to be addressed as the integration of all these factors. A consequence of this is that it becomes particularly evident where existing hypotheses are incomplete" (Webb, 2000). Contortions of shape, size, movement, and tension are key to most endothelial functions. Therefore, the cell's effectors (cytoskeleton and adhesions), generating the alterations in morphology, should be explicitly considered. Moreover, the subsequent sensory umwelt and collective feedback from such alterations should be made explicit (Figure 2D) (Fraccaroli et al., 2012; Franco et al., 2013; Galbraith et al., 1998; Shasby et al., 1982). The situation is complex, however. Filopodia, for example, are crucial to sprouting in tip cell guidance (Gerhardt et al., 2003) and play a key role in the fusion of tip cells, required to create new vessel loops (Bentley et al., 2009; Lenard et al., 2013). Yet recent work indicates that functional tip cell migration does occur in their absence, albeit at a slower pace (Phng et al., 2013).

An in vivo/in silico study recently pointed to a role of EC filopodia persistence in sprouting, which has not previously been considered (Villefranc et al., 2013). The study indicated that VEGF-C/VEGFR-3 signaling increases actin-driven protrusion persistence in zebrafish embryo intersegmental vessel growth. Simulant cells with less persistent filopodia (their effectors) mimicked VEGF-C mutants in that sprouts only extended halfway up between the somites. These simulants no longer stochastically "overshot" local VEGF sources with persistent filopodia, which is required to get past local VEGF maxima halfway up.

### *Compare Potential Mechanisms*

Often we do not know the underlying mechanism that generates a cell's behavior or tissue-level phenotype. This is a perfect time to turn to simulants. Simulants can be designed with a variety of hypothesized mechanisms inside them and then let loose in a simulation of the tissue environment to see which generates the closest matching behavior. This is an approach that has yielded great insight and received high impact recently in cell
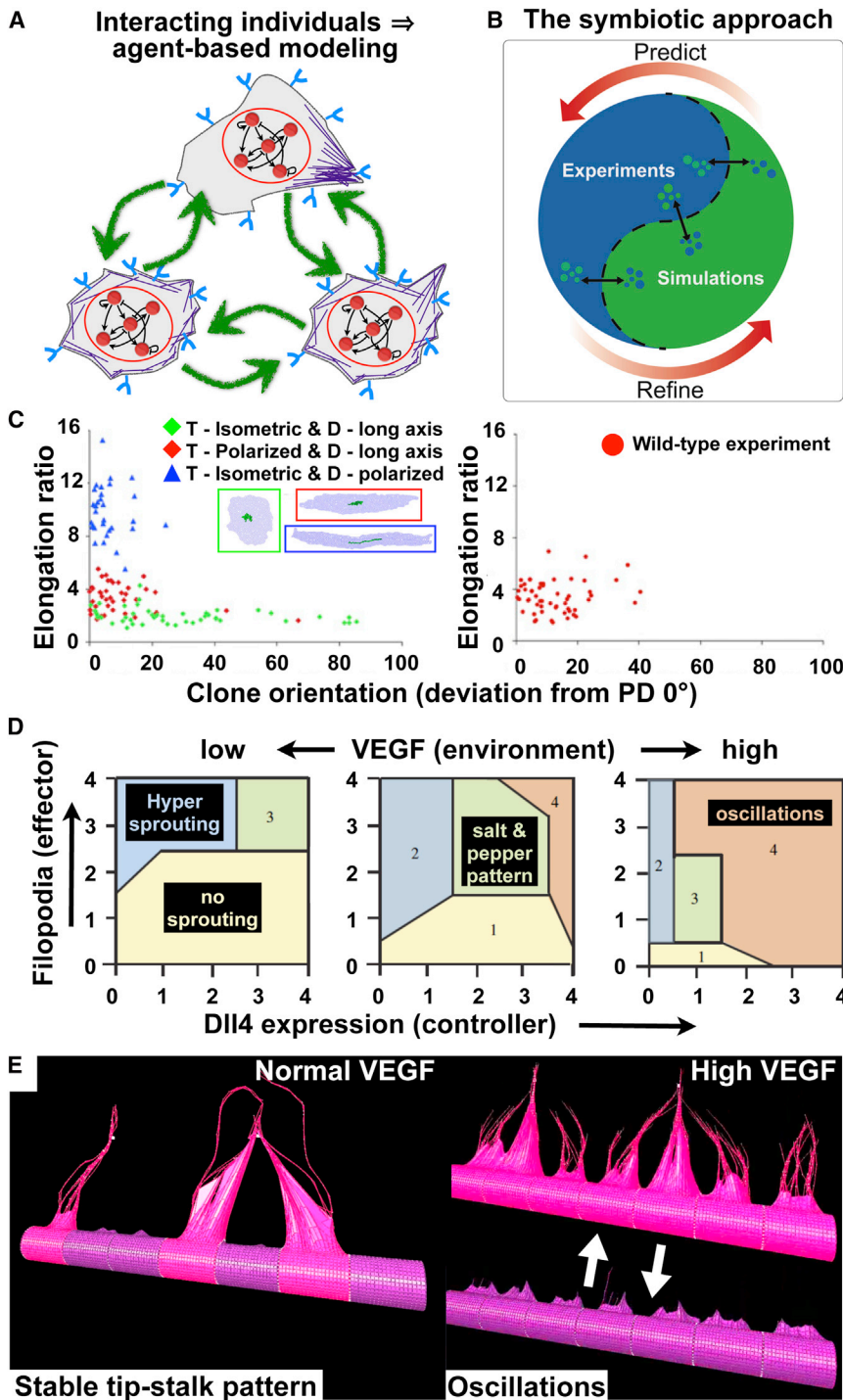
**A** Interacting individuals ⇒ agent-based modeling

**B** The symbiotic approach



**C**



Clone orientation (deviation from PD 0°)

♦ T - Isometric & D - long axis
♦ T - Polarized & D - long axis
▲ T - Isometric & D - polarized

● Wild-type experiment

**D** low ← VEGF (environment) → high



Dll4 expression (controller) →

**E**



Normal VEGF — Stable tip-stalk pattern

High VEGF — Oscillations

**Figure 4. Novel Biological Results Using Simulants**

(A) Agent-based modeling. Design autonomous simulants with a well-defined morphology, sensor placement, controller, and effectors. Situate them in an environment and compare the dynamics of their interactions to experimental data.

(B) Use the "symbiotic approach" between experimentalist and modeler to (1) iteratively refine the simulants design in light of new data and (2) test new insights from simulants experimentally. Reprinted from *Experimental Cell Research* (Bentley et al., 2013), with permission from Elsevier.

(C) Comparison of cell shapes generated by collective, tissue-level dynamics between simulant cells driven by different mechanisms to experimental cell shapes identified the mechanism most likely to drive planar polarization in epithelial cells. Adapted from Mao et al. (2011).

(D) Phase-plane analysis. Covarying filopodia extension (effector: y axis), Dll4 production in response to VEGFR-2 sensor activation (controller: x axis) and the environment (VEGF conditions) showed that the system tends toward oscillatory behavior with high VEGF. Nevertheless, normal tip cell selection and branch spacing is achievable under any VEGF conditions by tuning the simulant's effector and controller (lower/higher effector and controller levels are required for normal branching in high/low VEGF). Reprinted from *The Journal of Theoretical Biology* (Bentley et al., 2008), with permission from Elsevier.

(E) Unexpected synchronous oscillations in a sprouting vessel emerged in "rogue simulants" exposed to high VEGF (pink, high VEGFR-2 signaling and active sprouting; purple, inhibited, low VEGFR-2 signaling). Reprinted from Bentley et al. (2009).

each mechanism were then calibrated such that they matched the behavior of cells observed in one experimental data set (distance traveled by tip cells in zebrafish ISV). These calibrated simulants were then placed in a new environment and arranged in a chimeric vessel to mimic a second in vivo data set, which quantified tip cell contribution of mutant cells in chimeric zebrafish. Only the effector persistence simulant mechanism could match this second set of data, indicating it as the most likely candidate for further experimental study.

*Combinatorial Mutation Studies*

The reductionist approach used in traditional biology is even more readily available in simulation. In vitro and in vivo, we can perform gain- and loss-of-function experiments to see the effects of one component gene. Imagine being able to do this with ultimate control, to reduce/increase (by any small or large increments) components of a system and then quantify the changes in individual cell- and tissue-level behavior that ensue. In simulants we can tweak any component by any amount, alone or in combination, across one or many cells in the system. There are a number of ways to visualize data

biology (Figure 4D) (Mao et al., 2011; Marcon and Sharpe, 2012; Tozluoğlu et al., 2013). Indeed, in vascular biology this approach was used in the previously discussed VEGFR-C/VEGFR-3 simulant study (Villefranc et al., 2013). Endothelial simulants were designed in this study with one of a number of mechanisms of action of VEGF-C: a mechanism that alters the simulant's controller directly (determining Dll4 production) and other mechanisms that alter their effectors (cytoskeletal dynamics). Simulants with

using this approach, the clearest being the "phase diagram." This approach was used to generate a plethora of new insights and predictions in Bentley et al. (2008), shown in Figure 4C, leading to ongoing experiments and new data (Guarani et al., 2011; Jakobsson et al., 2010; Villefranc et al., 2013; Bentley et al., 2014).

Similarly, we can perform sensitivity analyses by varying all parameters of the simulants and their environments to determine which are important and to validate the model itself. Most pertinently, if the system exhibits the desired behavior only when a parameter is in a very narrow range, with large changes to behavior from small changes to that parameter, it is possible that the system is just incredibly sensitive. However, given typical biological variability, it is more likely that this indicates the simulant model is wrong or missing some regulatory factors. A good modeler will test all parameters in this way to maximize confidence in the model and provide the greatest insight. For example, sensitivity analyses in a multiscale simulation of vascular network remodeling identified the atomic mass of molecules excreted on tissue surfaces and washed out with flow as influential on subsequent cellular pillar formation and intussusceptive remodeling (Szczerba et al., 2009). Further, sensitivity analyzes on simulant cells recently showed that lumen formation via a combination of vacuole aggregation and cell-cell repulsion is more robust to parameter variations than lumen formation via either mechanism alone, suggesting why a concert of mechanisms may be in place to drive certain morphogenetic processes in real cells (Boas and Merks, 2014).

### Rogue Simulants: Insights into Normal and Pathological Endothelial Behavior
Vascular pathology is rife with examples of abnormal endothelial- and vessel-level shape changes. Tumors are characterized by their abnormal leaky, bulbous vessels (De Bock et al., 2011; Nagy et al., 2010); abnormal arteriovenous shunt vessels short-circuit the network in hereditary hemorrhagic telangiectasia (Marchuk et al., 2003); nearly spherical glomerular malformations or tufts are observed alongside leaky dilated vessels in diabetic retinopathy (Figures 1L–1O) (Wallace et al., 1998). Single-cell-level switches that drive these complex pathological forms are beginning to be identified. For example, Notch4 normalization was shown to drastically reduce arteriovenous malformations (Murphy et al., 2012). Pathology itself can be seen as the shift from adaptive behavior to maladaptive behavior when internal or external conditions become too extreme (Rubin et al., 2008).

If we have built cell simulants implementing the key AS components, then it is possible for them to exhibit and predict new mechanistic switches generating maladaptive behavior in pathological environments. The Bentley et al. (2008, 2009) simulant ECs revealed a fundamental new switch in pathological vessel formation by researchers observing the emergent dynamics that resulted from placing well-validated simulants into a new, pathologically high VEGF environment. When the VEGF concentrations increased, the cells' sensory input became so high that their lateral inhibition feedback no longer generated a heterogeneous mix of migratory and nonmigratory cells. Instead, all cells maximally inhibited each other via Notch. However, with this came the loss of their VEGFR sensors through downregulation, which in turn reduced the Notch signaling, so all cells became uninhibited and migratory. Over time, this cycle of synchronized migration/inhibition along the vessel repeated, completely disrupting branching (Figure 4E). The model predicted for the first time a potential role for changes in individual cells' relative, temporal dynamics in determining abnormal branch patterns. The extended version of this model, with junctional adhesions included as effectors of cell rearrangement, further predicted that synchronization of Notch signaling in high VEGF will also synchronize junctional dynamics, halting functional cell overtaking. This prediction was then confirmed in vivo where synchronized VE-cadherin patterning was indeed observed along vessels in mouse models of high VEGF pathologies such as glioblastoma and oxygen-induced retinopathy (Bentley et al., 2014). Intercalation has been shown to lengthen tubes, maintaining a small diameter (Ribeiro et al., 2004), suggesting a cell rearrangement defect as predicted by the simulant cells may contribute to the thicken vessels found in high VEGF pathologies.

### Toward Unification of Disparate Experimental Data
Biological study is fraught with variation, complexity, and results that are difficult to verify. The phrase "in our hands" is unfamiliar to a computer scientist, but a well-worn phrase in biology, indicating the result's repeatability in a different lab cannot immediately be assumed. Of course, there are also a wide variety of theoretical models one can build, and how one puts them together affects the results. So, assuming we have a good model of biology (there are many guidelines about what makes a good model of a biological process, as well as pitfalls to avoid; see Webb, 2001, 2009), do we face an equivalent "in our model" problem? Fortunately, the technical side of this problem (the equivalent to lab-to-lab variations in experimental artifacts) can be readily avoided in modeling. When publishing, all parameters need to be published along with exact details of model building, leaving no room for variation in results between labs (especially as code should also be published). The more enlightening case is when two different models of the same biological behavior disagree in their predictions. In this case, one can "lift the hood" of the two models, compare the main elements in each, and see why they differ. Indeed, it is often possible to transform one model into another in an element-by-element way, with the change of results proving very informative (Husbands et al., 2010). Thus, the "in our model" problem actually becomes an asset for pinpointing hidden assumptions that can impact their predictive power.

Simulant cells could thus help us untangle the mess of apparently contradictory results, or the effects that different assays may have on behavior, by considering the situated and embodied effects of cells in different environments and geometries. For example, cells in a sprout experience other cells and the environment in a fundamentally different, spatial way compared to cells in a 2D monolayer. Moreover, in vivo quantification techniques have their own limitations. Counterintuitive results can be explained by simulating different scenarios and comparing the in vivo quantification method with all aspects of the model directly outputted, aspects of the simulants, which would not be possible with real cells. For example, the simulation study by Stefanini et al. (2010) identified a mechanism to explain the counterintuitive result that anti-VEGF therapy elevates VEGF levels in the blood. Observation methods also vary from group to group, given the high level of hand-quantified data and subjective determination of how to quantify them. Measurement techniques can vary, and conclusions, even significance drawn from the same data, can be fundamentally different between labs. By simulating different quantification methods, we can confirm that differences

between labs' observations may be due to the quantification technique, not differences in the underlying behavior, as explored in Villefranc et al. (2013) and Bentley et al. (2014).

## Concluding Remarks

Do EC (simulants) dream of eclectic shape? How shall we now answer this question? Perhaps it is most fruitfully asked of the experimentalist reader, as it is asked of the reader in Philip K. Dick's novel *Do Androids Dream of Electric Sheep?* In the novel the reader has traveled alongside the rogue android hunter Deckard as he is exposed to the latest simulant's capabilities and their desire to live—to be treated equally. At each turn, the reader is forced to question their assumptions and wonder: could simulants be capable of emergent, life-like, adaptive, and unexpected behaviors? Can greater integration of simulants, with experimentation, teach us something new, something fundamental about the very nature of living systems? The challenge is ahead: can we overcome cultural differences and realize simulants' emerging potential in cell biology? Do *we* dare to dream?

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