

Bridging Structure and Process in Developmental Biology through New Imaging Technologies

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Many unexpected discoveries in developmental biology have depended on advancement of imaging technologies to visualize developmental processes as they unfold across multiple spatial and temporal scales. This essay surveys the recent advances in imaging, highlighting emerging capabilities with an eye toward those poised to have the greatest impact on developmental biology.

It is a remarkable aspect of animal development that cells within an organism harbor virtually identical genes, yet the organism is capable of growing from a single cell into a multibillion cell system with diverse cell types exhibiting complex behaviors. In the past, it has been possible to group the approaches that researchers take in studying how organisms develop into two basic camps. One approach is focused on structure, delineating the epigenomic state of the developing organism to obtain the blueprint of normal development, down to the level of individual genes and their control pathways. The second approach is focused on process, characterizing the cell's physical and chemical relationships with its environment (including with other cells, tissue matrixes, and blood) during the organism's development. This dichotomy of structure and function goes back to the ancient Greeks—is nature made of static structures or processes? It would be an overstatement to say that these two approaches in contemporary biology have been hermetically sealed, but increasing crosstalk over the past ten years is achieving more rapid advances toward a comprehensive understanding of developmental biology.

Consider how organs, including heart, stomach, and liver, acquire left-right asymmetry within a developing organism (Lee and Anderson, 2008). Using the structure-centric approach, particularly based on molecular biology, researchers have tackled this question by analyzing gene deletions in organisms where left-right asymmetry was lost. The affected genes included those coding for intraflagellar transport, kinesin motor activity, and planar cell polarity signaling compo-

nents. How these molecules contributed to the development of left-right asymmetry was unclear based solely on this structuralist approach. But using the process-centric approach to examine, by imaging, the integrated activities of these molecules, researchers soon linked the mutated gene products to leftward fluid flow mediated by monociliated cells distributed across the developing node in the embryo (Hirokawa et al., 2006). The circular beating of the cilia on nodal cells was found to be key to the initiation of asymmetric organ development, either through the movement itself or through sweeping signaling molecules to one side of the nodal region. This explained the requirement for intraflagellar transport and kinesin activity, since they are needed for ciliogenesis. Moreover, the specific positioning of cilia to the posterior region of the apical domain of nodal cells helped explain the requirement for planar cell polarity components.

Too often in the past, researchers have assumed that a protein's physiological or developmental role could be revealed simply by knocking down the protein of interest and observing the resulting phenotype. As the above example illustrates, the mechanistic link between protein and phenotype is not always obvious. This is because most developmental processes arise from changes in the physicochemical relationships among cellular materials, such as cytoskeleton and membranes, with discrete physicochemical characteristics giving them mechanical and biophysical properties. Integrated relationships among these materials give rise to key properties of the cytoplasm and its membranes. These, in turn, connect with mechanical and biochemical

signals, working to position cells within multicellular tissues, deliver environmental information, and allow cells to control their surroundings. Simply summing up gene product outputs in such a system, therefore, has not provided sufficient mechanistic insight into the system's behavior as a whole.

That said, valuable information has been obtained with a structure-centric approach aimed at obtaining an understanding of how each cell in a developing organism acquires its unique pattern of gene expression and epigenetic variation, with specific genome-side patterns of DNA methylation, histone modifications, transcription factor binding, and chromatin compaction that determines which regions are transcribed. But pinpointing essential gene-based modifications and products in this fashion does not, by itself, bring full knowledge of various facets of development. Organ and tissue phenotypes result from numerous complex interactions within and among cells, with feedback loops, self-organizing capabilities of molecular machines, and diffusion barriers all playing roles in how a gene product functions (Friedl and Zallen, 2010). Without insight into the engineering principles underlying such cell organization and function, the task of connecting genotype and phenotype is daunting. This is even more so because many cell and organ processes are deeply entwined in cell physiology and metabolism, which until recently had largely gone out of style as fields of study because of a molecular biological and structure-centric focus (McKnight, 2010).

Therefore, in a variety of areas a fuller understanding of how developmental processes and organization are related

has drawn on both a structure-centric approach employing molecular genetics (to reveal molecular, gene-based inputs) and a process-centric approach employing imaging (to reveal dynamic relationships). Looking forward to the next ten years, we can expect that more ways will be found to bring researchers employing these two approaches together for constructive synergy. As has been true to date, it is likely that the advancement of ever more powerful imaging technologies that facilitate investigation of both structure and process in the study of animal development will play a central role in this endeavor.

Merging Structure- and Process-Centric Views through Imaging

Light microscopy imaging technology makes it possible to bridge structure- and process-centric research strategies because of its ability to provide quantitative descriptions of spatiotemporal relationships among structural determinants and outputs associated with cells and tissues. These descriptions can then be used for building and testing models of developmental processes and their design principles. Many key discoveries in developmental biology over the past ten years have benefited from this approach, often revealing unexpected cell behaviors underlying tissue function, organization, and development. For example, 3D time-lapse imaging of organotypic cultures to observe epithelial morphogenesis has revealed novel roles of collective cell migration and heterotypic cell interactions (Ewald et al., 2008). In addition, mechanical inputs from physical forces have been shown to act as signals that influence gene expression, modulate cellular processes, and control tissue organization (Kobayashi and Sokabe, 2010). Moreover, morphodynamic processes, including cell elongation, polarization, and contraction, have been shown to underlie processes as diverse as epithelial closure, tissue elongation, and nervous system morphogenesis, as well as stem cell maintenance and tumor progression (Skoglund and Keller, 2010). These new discoveries, while dependent on genetic and biochemical approaches to identify new molecules, were only possible as a consequence of seeing underlying relationships through multidimensional imaging.

Ongoing advances are driving this ever-expanding use of light microscopy imaging in developmental biology. Progress in multiple technological fronts is permitting experimental capabilities for interrogating developmental systems across multiple spatial and temporal scales. Improvements in microscope systems allow probing of fine ultrastructure or visualization of cellular dynamics in whole organisms during development. Advances in automation and image analysis, furthermore, are enabling rapid screening and large-scale anatomical reconstruction. These achievements have come from an expanding set of fluorescent markers, functional indicators, and genetic strategies for fluorescent labeling, as well as improvements in optics and computational techniques.

Advances in Fluorescent Protein Technology

The increased availability of fluorescent markers for visualization has been particularly impressive. Foremost in significance is the genetically encoded green fluorescent protein (GFP) from *Aequoria Victoria* and its relatives (Tsien, 1998). These proteins can be fused to virtually any protein of interest and used in different microscopy techniques to visualize cellular processes on many spatial scales. The fluorescent fusion proteins are easily constructed, show specific targeting, and are minimally perturbing to a biological specimen, unlike early approaches using fluorescent antibodies or exogenous dyes. Their high sensitivity, resulting from production of light of a different color from the illuminating light, allows cellular processes to be accurately monitored over seconds, minutes or days. Laboratory mutagenesis has diversified GFP's spectra, increasing its brightness and folding efficiencies as well as producing different colors, which allow for simultaneous imaging of multiple sets of proteins inside cells (Shaner et al., 2007). Mutagenesis has also led to the generation of forms of GFP that are photoactivable or photoconvertible, which make it possible to highlight specific protein populations to examine turnover and fate mapping (Lippincott-Schwartz and Patterson, 2009). Finally, fluorescent proteins (FPs) from marine corals have been mutated to produce a series of red-shifted proteins useful in deep tissue imaging due

to their long wavelengths (Fradkov et al., 2000).

The accessibility of such engineered FPs with different colors and behaviors has led to the emergence of a whole field of specific experimental strategies to clarify spatial compartmentalization and temporal dynamics of proteins. Among the imaging techniques having quantitative impact are fluorescence recovery after photobleaching (FRAP), photoactivation, fluorescence correlation spectroscopy (FCS), fluorescence resonance energy transfer (FRET), and fluorescence lifetime imaging (FLIM) (Lippincott-Schwartz et al., 2003; Miyawaki, 2011; Digman and Gratton, 2011). In each case, changes in the FP's signal in a specified area in the cell give insights into the fusion protein's diffusion, binding/dissociation kinetics, lifetime, conformational changes, and/or intermolecular interactions. This has allowed researchers to interrogate and quantify protein interactions and relationships in cells and tissues in unprecedented ways. While caution is always needed to ensure that the FP tag is not affecting the protein's behavior, it is remarkable how many different proteins tagged with FPs show identical behavior to their endogenous counterparts.

The new information about protein behavior and dynamics within cells obtained from these imaging techniques has been highly beneficial for deciphering the complex pathways driving cell and developmental processes.

One example is in the area of signal transduction, where FRET-based approaches are allowing the monitoring of regulatory interactions between signaling molecules (Mehta and Zhang, 2011). FRET allows detection of protein interactions less than or equal to 100 Å (dependent on energy transfer from donor to acceptor for signal creation) in real time in live cells. Consequently, inter- and intramolecular distances associated with proteins can be probed, as well as transient protein-protein interactions over short time periods (often missed in classical biochemical approaches requiring large isolatable fractions) (Miyawaki, 2011). By placing a conformationally sensitive protein, such as a genetically encoded calcium or PKC activity reporter, between a FP FRET pair, key information has emerged for understanding how signaling molecules interlink as circuits to

control dynamics of signal flow (Mehta and Zhang, 2011). In addition, input of the data into mathematical models has helped uncover complex features of signaling pathways, including negative feedback, bistability, and oscillatory signaling dynamics.

In addition to reporting on a protein's dynamics, FPs can be used as biosensors for detecting different cell states (Zhang et al., 2002). Recent probes in this category include those for monitoring GTP hydrolysis, calcium signaling, and cell cycle events. FP probes also have been designed to perturb discrete biochemical activities. By changing a protein's distribution or interactions, these probes allow specific biological activities to be altered at selected times and places in cells. One strategy includes FPs modified so they bind small molecules capable of dimerizing, which triggers a change in the protein's behavior (Karginov et al., 2010). Another exciting approach involves optically inducible switches, which employ light to discretely activate signaling molecules (Gorostiza and Isacoff, 2008).

Coupling of genetically encoded targets with synthetic fluorophores much smaller than FPs offers the possibility of marking proteins that would otherwise mistarget or fold incorrectly when fused to a FP (Fernández-Suárez and Ting, 2008). In this approach, a peptide or protein sequence capable of recruiting a small synthetic fluorescent molecule is typically expressed in living cells. Techniques where this has proved successful include SNAP tags (Campos et al., 2011) as well as those known as FIAsH and ReAsH (Machleidt et al., 2007). In FIAsH and ReAsH, addition of a small fluorescent molecule to bind to a cysteine residue engineered into the genetic target lights up the target, allowing its dynamics to be imaged. Using ReAsH, it is possible to perform correlative light and electron microscopy (EM) due to its ability to generate a specific photooxidation reaction that yields an electron-dense signal visible in the EM.

Small molecule fluorescent probes are also being used in reporter technologies for probing native biochemistry of metabolites, including ions such as zinc and nitric oxide, which drive numerous physiological processes, or, when uncontrolled, trigger pathology (Zhang et al., 2002; Pluth et al., 2011). The zinc indica-

tors typically are intensity-based sensors, usually associated with fluorescein, responding to zinc coordination with an increase in fluorescence emission intensity. Nitric oxide probes, on the other hand, include those in which the oxidation product of NO reacts with a functional group to modulate its fluorescence. Using these and other indicators, the generation, accumulation, and translocation of key metabolites are being studied with spatial and temporal resolution, revealing how they respond to specific inputs (Pluth et al., 2011). This is bridging structure and process approaches, by clarifying the ways in which the multiple enzymes and pathways known to utilize organic species are interconnected and regulate diverse aspects of biological systems.

Advances in Microscopes: Diffraction-Limited

The present generation of light microscopes has been modified in nearly all parameters compared to similar microscopes of only a decade ago, enabling imaging over unprecedented spatial scales and experimental situations. Due to key improvements, it is now possible to obtain speeds of image acquisition of ~ 120 images/s or even higher, and to have multispectral imaging due to minimization of spectral emission overlap. Microscope systems incorporating these modifications include commercial light scanning confocals, spinning disk confocals, and wide-field microscopes with total internal reflection. Many of these systems have built-in macros for performing kinetic experiments such as FRAP, FRET, or FCS. Advances in automation and image analysis are additionally making it possible to do rapid screening and large-scale anatomical reconstruction using these microscope platforms.

In addition to having brighter lasers and faster imaging, the modern confocal and spinning disk systems are capable of irradiation of specific areas of a specimen. This allows researchers to selectively photobleach or photoactivate a specimen. By highlighting discrete pools of a protein population in this manner, it becomes possible to visualize and quantify the protein's overall steady-state dynamics, including its turnover kinetics and trafficking pathways. Often, surprising characteristics are observed, such as the rapid association/dissociation kinetics

of proteins associated with membrane coat complexes and the nucleolus (Lippincott-Schwartz et al., 2003). These dynamics were not apparent in the steady-state representations of the proteins obtained from conventional imaging or biochemical fractionation approaches. The knowledge obtained is pulling together structure and process camps, by revealing how macromolecular structure relates to assembly, flow, and turnover of components.

Impressive technological innovations of modern microscopes also extend to the study of whole, living organisms. Conventional confocal microscopes usually allow imaging of no more than 44 μm deep into a tissue due to light scattering. But many important processes relevant for understanding tissue and developmental function occur deeper than this, so scientists are working to push the depth resolution capabilities of microscopes. A powerful approach for achieving increased depth penetration into a specimen is two-photon microscopy (Helmchen and Denk, 2005). It uses near infrared illumination, which goes deeper than visible light, to convert two or more incoming photons into an outgoing photon of distinct color. The spatial confinement of the excitation volume permits imaging deep into a specimen with inherent optical sectioning. To allow imaging of depths in the centimeter range into tissues, two-photon imaging can be combined with microendoscopy, which employs a microendoscope comprised of a thin but rigid optical probe that inserts into tissue to conduct light to and from deep tissue locations (Flusberg et al., 2005). By scanning a laser focal spot outside the tissue, the probe device projects and demagnifies the scanning pattern to a focal plane inside the tissue. In this way, it becomes possible to explore cell properties in the context of the whole organism, such as in the cavities of internal organs or in the pathways of blood capillaries (Monfared et al., 2006).

Plane illumination microscopy offers a further exciting possibility for in vivo volumetric fluorescence imaging (Huisken et al., 2004). In this approach, illumination comes from a sheet of laser light 2–8 microns thick produced by a cylindrical lens, usually of modest numerical aperture (NA) and long working distance. Optical sectioning is accomplished by turning the sample in different directions to allow

the laser sheet to illuminate successive planes. This enables very thick specimens, including whole intact embryos, to be imaged completely with high speed and low light exposure, as shown in an elegant study of the gene and protein expression patterns of the developing *Medaka* fish embryo imaged over several days (Keller et al., 2008). In addition to embryonic development, successful applications employing plane illumination microscopy include studies involving anatomical mapping, particle tracking, and functional imaging of neural activity (Holekamp et al., 2008).

Because the thickness of the light sheet in plane illumination microscopy diverges greatly over the field of view, the technique has until recently been limited to the multicellular, micron-level domain. However, with the use of Bessel beams to create thinner light sheets, it is now possible to extend plane illumination microscopy to the subcellular, nanometric-level domain (Planchon et al., 2011). Creation of the Bessel beam is accomplished by positioning an annular apodization mask in front of the excitation objective. This creates a thin light sheet of less than 0.6 μm that can be scanned rapidly over $60 \times 80 \mu\text{m}$ fields of view. The resulting 3D high-speed live cell imaging (i.e., 10 ms per image plane) is unprecedented and can provide astonishing time-lapse sequences of 3D organization within and between cells. This advance promises to be highly influential in clarifying many aspects of the dynamics and relationships of cell interactions within complex tissues that have eluded other methods such as two-photon and traditional light sheet planar microscopy because of their limited z resolution and slower optical sectioning speeds.

Another area in deep tissue imaging undergoing dramatic improvements is fluorescent signal detection. Diffraction-limited imaging is rarely achieved deep inside thick specimens because of optical distortions. These arise as excitation and detection pathways are aberrated by refractive index inhomogeneities within the sample. New approaches in the field of adaptive optics are helping to correct this problem (Booth et al., 2002). One strategy uses segmentation of the rear objective lens, allowing significant improvement of signal and spatial resolution

at depths up to 400 μm (Ji et al., 2008). Used in conjunction with optical clearing reagents to further alleviate light scattering within tissues, even better resolution capabilities are expected.

These various improvements in deep tissue imaging are highly relevant for bridging the two camps of structure and process. By providing better visualization of the unfolding of developmental processes in a living organism, the improvements allow appreciation of new principles such as how mechanical forces and tissue environment function in determining cell phenotype. These are challenging to assess from examining patterns of gene expression and epigenetic variation alone. As a specific example, tissue imaging of migrating cells during cancer progression has revealed that cells shift migratory styles, from mesenchymal-like to more rapid amoeboid-like, due to accompanying changes in the cancer cell and tumor microenvironment (Wolf et al., 2007). This suggests that a cancer cell's environment strongly affects its epigenetic state (Weigelt and Bissell, 2008), a reversal of the common notion that epigenetic state primarily controls cell phenotype.

Advances in Microscopes: Superresolution

Until recently, optical resolution below $\sim 200 \text{ nm}$ in x-y and $\sim 500 \text{ nm}$ in z has been impossible due to the diffraction limit of light. This has hampered the study of many facets of developmental biology arising over small length scales, such as molecular processes in small structures such as tight junctions synapses, microfilaments, and nuclear pores. Advances in super-resolution microscopy are changing this, enabling optical examination of nanometer-scale phenomena. One strategy for pushing the limits of spatial resolution employs stimulated emission to narrow the focal spot of the microscope. Called stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994), this technique uses a pair of overlapping concentric laser beams scanned together, with the first beam exciting fluorophores lying within a diffraction-limited spot and the second beam using stimulated emission to narrow this spot by preventing fluorescence at its periphery. STED microscopy can typically achieve 10-fold higher resolution than

conventional fluorescence imaging, allowing new insights into topics as diverse as tracking synaptic vesicles in neurons, monitoring shape changes in dendritic spines, and measuring lipid dynamics in the plasma membrane (Nägerl et al., 2008; Eggeling et al., 2009). Another approach for breaking the constraints of diffraction is saturated structured illumination microscopy (SSIM) (Gustafsson, 2005; Heintzmann et al., 2002). It achieves this by illuminating the sample with a sequence of periodic patterns of high spatial frequencies that can reach saturating excitation intensities. Fine spatial details in the sample at less than 100 nm resolution are then extracted computationally from the raw images using deconvolution algorithms and Fourier transformations (Schemmelleh et al., 2008).

Still higher resolution has been achieved with the introduction of single-molecule-based super-resolution techniques (Patterson et al., 2010). These approaches exploit the stochastic activation of fluorescence to detect and localize single fluorophores within dense populations. Photoactivated localization microscopy (PALM) (Betzig et al., 2006) employs photoconvertible fluorescent proteins to accomplish this, whereas stochastic optical reconstruction microscopy (STORM) relies on photoswitchable dyes (Rust et al., 2006). In both approaches, structures labeled by an ensemble of photoconvertible molecules too dense to be imaged simultaneously can be resolved with nanometric precision, providing finer spatial resolutions to cellular structures than has been previously possible with light microscopy. Although electron microscopy can still provide images of finer ($\sim 1 \text{ nm}$) resolution than those ($\sim 20 \text{ nm}$) regularly produced by these techniques, because PALM/STORM can pinpoint the localization of tens of thousands of fluorescent proteins precisely targeted to subcellular structures, they offer greater possibilities of untangling molecular relationships, stoichiometry, and cluster characteristics of proteins (Patterson et al., 2010). This is important for bridging the dichotomy of structure and process approaches since it permits the spatial ordering among proteins to be determined and related to their functions. For example, in an interferometric PALM approach providing 10 nm z resolution (Shtengel et al., 2009), the functional

architecture of focal adhesions (the “feet” allowing cells to interact with the extracellular matrix via integrin receptors) was mapped out by precise localization of different adhesion components relative to each other and the substrate (Kanchanawong et al., 2010).

Data Analysis and Hypothesis Testing

As light microscopy imaging has advanced over the past decade, so have the approaches for collecting and analyzing its data. Image data sets of many types now require extensive, often model-based, computational analysis just to be interpreted. This is because the basic characteristics of the data provided by the light microscope have changed dramatically. Due to the use of digital image acquisition cameras, images are typically provided in numerical format, with a specified number of bits per image pixel. To analyze an image, therefore, requires image data analysis tools, in which the representations of a sample are reconstructed computationally. In FCS, for example, intensity fluctuations resulting from migration of fluorescent objects into and out of a small volume are analyzed mathematically and correlated to reveal their size, speed, and interactions (Digman and Gratton, 2011). Even in images obtained from regular confocal microscopes, the data are digitized and the underlying biological reality is reconstructed computationally. Because images are created on the basis of relationships among numerical pixel outputs, researchers need to be especially cognizant of their underlying assumptions in interpreting the data (Wilt et al., 2009). The data themselves fall within neither the structure nor process camps and it seems most productive to use a synergistic combination of hypotheses focused on structure and process.

Future Outlook

Major breakthroughs in imaging are occurring in multiple technological fronts, impacting the developmental biologist's ability to examine the nanoscale, to create large-scale tissue reconstruction, and to image cellular properties of live animals. Many methods are still in their early stages of development but as these approaches mature, we should expect to see ever more sophisticated combina-

tions of complex fluorescent labeling strategies with in vivo or superresolution microscopy. By allowing visualization of processes and relationships within and between cells, imaging techniques are confirming that it is not just the epigenetic expression pattern or structure that is responsible for the physical properties of a developing organism. Equally important are the relationships among gene products, which produce complex, self-organizing patterns of activities. Utilizing the increasing menu of imaging techniques, highly collaborative investigations of these processes, and their underlying structural elements, are providing key insights into how an organism develops and functions.

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