Movies, measurement, and modeling: the three Ms of mechanistic immunology

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Immunological phenomena that were once deduced from genetic, biochemical, and in situ approaches are now being witnessed in living color, in three dimensions, and in real time. The information in time-lapse imaging can provide valuable mechanistic insight into a host of processes, from cell migration to signal transduction. What we need now are methods to quantitate these new visual data and to exploit computational resources and statistical mechanical methods to develop mechanistic models.

For centuries scientists have sliced, homogenized, and resuspended biological tissues to probe the inner workings of the cell and its host. The isolation of cells from the in vivo environment reduces the enormous complexity of life to a manageable minimum and permits the controlled manipulation of a system of interest. In vitro methods have largely driven the explosive progress in the life sciences, advancing biology from the days of Needham's 18th century theory of the "life force" and spontaneous generation to today's modern era characterized by numerous subdisciplines.

However, the study of biological systems outside of the in vivo setting has its drawbacks. Biological processes depend on the interplay between numerous elements that constitute a microenvironment rich in complexity. It is impossible to fully mimic this complexity outside of the host tissue. Also, events in vivo are often associated with a rare subset of cells contained within a population. Important information concerning the frequency and identity of these relevant cells is missing in biochemical approaches, such as PCR, which assay for the activity of interest by averaging over the entire population. Moreover, biological processes are never static. At the population, single cell, or molecular scale, biologically pertinent events involve dynamic events in three spatial dimensions. But most experimental approaches do not investigate the spatiotemporal evolution of a system in four dimensions. Hence, they can only provide partial or static glimpses of dynamic processes.

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Recent advances in imaging technology have enabled scientists to overcome these limitations and visualize cellular and molecular processes in three dimensions in real time. We can now probe the subcellular world with nanoscale resolution and can even peer into the depths of live tissue to witness events taking place in the in vivo setting. These capabilities represent a powerful experimental achievement and have the potential to revolutionize the study of the life sciences in general, and the field of immunology in particular.

Imaging in living color

Live intact tissues can be imaged using two-photon laser–scanning microscopy (TPLSM) either intravitally with the animal anesthetized or by isolating and imaging tissue explants maintained in oxygen-supplemented media at controlled temperature (1–5). Using a femtosecond pulsed-emission laser and near infrared light for excitation, optical slices are acquired at descending depths (along the z axis) of up to several hundred microns into the tissue generating a stack of images. These z stacks are acquired in quick succession at regular time intervals. With the aid of computer software, the z stacks are rendered into three-dimensional images, which are then displayed as a contiguous stream in fast succession. The results provide high resolution time-lapse video of cellular behaviors deep inside living tissue.

Similar time-lapse images of live cells can be obtained using other types of imaging such as epifluorescence microscopy (combined with deconvolution) and confocal microscopy—technologies that do not, however, allow in-depth imaging of tissue (6). Images captured by epifluorescence and confocal microscopy provided our first glimpses into the process of immune recognition (7–11) and gave rise to today's intense investigation of the formation, structure, and function of the immunological synapse (12).

In addition to revealing new behavior at the tissue and cellular level, recent improvements in imaging technology are allowing scientists to visualize processes with nanoscale resolution, revealing intracellular activity which has never before been imagined, much less seen. Recent work by Kindzelskii and Petty using epifluorescence microscopy captured time-lapse images of intracellular calcium flux in neutrophils upon stimulation (13). These vivid, patterned waves of calcium could only be visualized by acquiring images at submicrosecond resolution. There is no doubt that the ability to visualize events at the level of individual cells with this kind of spatiotemporal resolution will have a radical impact on the study of signal transduction, which until now has been dominated by biochemical study of isolated cell populations rather than direct observation of single cells.

Be it on a molecular scale or at the tissue level, being able to visualize events in real time reveals new information concerning the spatial distribu-

tion and the dynamics of cellular events that simply cannot be gleaned from conventional experimental approaches. But with such huge strides in technological development come new challenges. Although advanced imaging technology can indeed produce stunning images, the data sets provide far more than entertainment for the scientific community. There is a wealth of information contained in visual data that reflect mechanisms underlying the function of biological systems. The challenge we face now is to develop the means to extract mechanistic understanding from the data and thereby take full advantage of the powerful capabilities afforded by today's new age of scientific imaging.

We believe that two approaches will be the keys to mining the wealth of information in data obtained from imaging experiments. The first is to develop methods to quantitatively analyze the data. The second is to develop models that can be simulated on a computer and can be used to test hypotheses for mechanisms underlying the observed behavior.

Quantitating the visual. Properly done, quantitative analysis of data can provide a precise description of visual information that cannot be conveyed by subjective language. For example, the statement that "T cells move faster than B cells when migrating through the lymph node" is unsatisfying, leaving open questions such as, "How much faster?" and "Do T cells move faster all the time or only during certain time intervals?" With the recent release of specialized computer software, Imaris, developed by Bitplane, it is now possible to track fluorescently labeled objects, either cells or particles, moving in three dimensions through time (Fig. 1; reference 14). With this new capability comes the means to make quantitative and time-resolved measurements that can precisely characterize motility in terms of velocities (speed and direction of movement), acceleration, and deceleration. Importantly, this software is user friendly, and Bitplane offers both personalized and Web-based training sessions.

The ability to assess differences and similarities with precision provides the necessary objectivity to unambiguously interpret experimental results. For example, cells migrating through tissue exhibit a characteristic degree of pausing (Fig. 2), which can be measured (2). Quantitative information about cell movement can sometimes be used to test predictions as to what would happen to this behavior if the system were manipulated (for example, the loss of a critical adhesion factor involved in thymocyte-stromal cell synapse formation). We can predict that under these conditions the frequency and/or duration of migratory pausing will decrease and we can experimentally determine the developmental consequences of such a change. Moreover, the significance of observed differences can be evaluated using statistical methods that can establish confidence limits on the data and its interpretation.

Quantitating visual data removes the subjectivity that can sometimes taint interpretation of qualitative observations. When viewing time-lapse video, one's attention can easily be drawn to a behavior that "catches the eye," possibly leading to the erroneous conclusion that the behavior is representative or otherwise significant. Computing the frequency of such a behavior may reveal that it is extraordinarily rare and, despite the eye's tendency to focus on it, may not be biologically meaningful. Conversely, it is also possible that rare events that direct biologically important phenomena can be masked by the dominant and uninteresting dynamic events.

Quantitative measurements can also lead to totally new mechanistic insights that would simply not have been revealed without the precise data. Davis and coworkers have developed single molecule-counting methods where individual agonist peptide–MHC molecules can be fluorescently tagged, and their numbers at the junction between a T cell and an APC can be precisely determined. This enabled them to show that $CD4^+$ T cells undergo transient calcium signaling upon stimulation by a single agonist peptide–MHC molecule, and that sustained calcium signaling and immunological synapse formation can be induced by as few as 10 agonist peptide–MHC molecules in a sea of endogenous peptide–MHC molecules (~30,000) in the contact

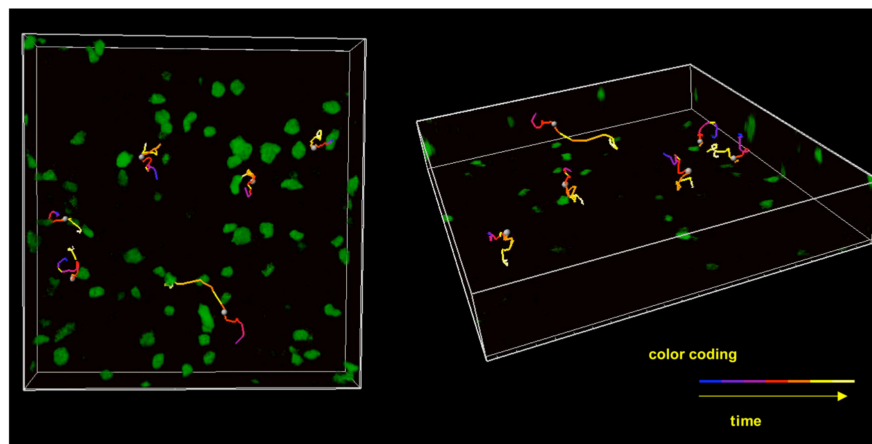

Figure 1. Tracking cell migration in four dimensions. A still photo taken from a time-lapse video of GFP-labeled thymocytes moving in three dimensions through an intact thymic lobe acquired by TPLSM. The image (left) was reconstructed from a single z stack and is shown along the x and y axes with selected migratory tracks highlighted. Tracks were generated using Imaris computer software by Bitplane, and are color coded to indicate the passage of time from blue (beginning of imaging) to yellow (end of imaging). The volumes of tracked cells have been hidden to better show the tracks. The x, y, and z coordinates of the tracked object are determined at each time point throughout tracking. Coordinate data is then imported into Microsoft Excel and can be used to extract a number of measurements related to three-dimensional movement. (Right) The same image has been rotated along the z axis. All cell volumes have been hidden.

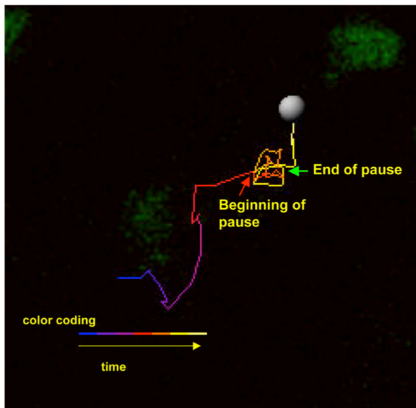

Figure 2. Quantitating migratory pausing. A still photo taken from time-lapse video acquired by TPLSM shows the migratory path taken by a single GFP-labeled thymocyte as it moved through an intact thymic lobe. The red arrow indicates the point at which cell displacement ceased and the green arrow indicates the time at which the cell resumed its displacement. This behavior can be measured to determine the incidence and the average duration of pausing calculated from thousands of migratory events (unpublished data).

zone (15). These observations are in contrast to the previous supposition that CD4$^+$ T cell responsiveness required on the order of 60–400 agonist peptide–MHC molecules (16–19). This previous estimate had been derived from biochemical methods that calculated the average number of agonist peptide–MHC complexes that an APC expressed on its entire cell surface. Quantitation of the direct visualization of molecular events in the contact zone provided a precise value for the minimum number of agonist peptide–MHC molecules required for stimulating T cell signaling.

This finding of an unexpected sensitivity and selectivity of T cell response to antigen raised many important mechanistic questions such as: how is the extraordinary sensitivity with which T cells detect "self" from "nonself" not accompanied by frequent false positives? This question, motivated by precise quantitation of direct visualization experiments, led to synergies between mathematical models and biochemical and imaging experiments that have suggested striking new models for early activation events in CD4$^+$ T cells (7, 15, 20). In particular, the suggestion (15, 20) that the formation of dimers of self and agonist peptide–MHC molecules and the spatial localization of a kinase (Lck) play key roles in amplifying the sensitivity and selectivity with which T cells detect antigen provides a framework for future experimentation.

Quantitating some types of visual data can be straightforward, as in the case of measuring migratory speed with computer software assistance. Quantifying more complex phenomena, such as the dynamics of changes in cell shape, is more challenging and will require collaborations between immunologists and physical scientists and applied mathematicians.

Modeling cooperative dynamic processes. Intracellular signaling and effector functions resulting from receptor–ligand binding are emergent properties that involve cooperative events and many cellular components. The migratory behavior of cells in the thymus or lymph nodes is also influenced by many events occurring in concert. Direct visualization experiments are providing vivid images of the consequences of these cooperative dynamic phenomena. The inherently cooperative nature of dynamic processes in cell biology, however, can confound our ability to understand mechanism based on observations of just a few experimental reporters. Statistical physics is a discipline that has been focused on understanding collective dynamic processes in synthetic systems. As we focus on developing a mechanistic understanding of cooperative dynamic processes in immunology and cell biology, it seems that the time is ripe for synergies between statistical mechanical model building and genetic, biochemical, and imaging experiments. Recent studies have shown that such a marriage between in silico, in vitro, and in vivo studies can be fruitful in developing mechanistic insights (20–23). Counterintuitive ideas regarding the signaling function of the immunological synapse, the importance of spatial organization of receptors, ligands, and kinases for the extraordinary sensitivity of T cells to antigen, mechanisms underlying TCR down-regulation, and signaling by the Fc receptor are just some examples of insights that have emerged from such synergies between methods rooted in the physical and biological sciences.

Mathematics, physics, and the future of biology. Mathematics is well appreciated as being the language of physics, elegantly and concisely describing physical phenomena over a wide spectrum of length and time scales. Understandably, the disciplines of physics and mathematics have often developed in concert, leap-frogging over one another as an achievement in one field led to the advancement of the other. Indeed, the development of one of the most influential branches of mathematics, calculus, was partly driven by the physicist's desire to explain planetary motion (24). With some notable exceptions (25), the histories of biology and the physical sciences have been far less intertwined. But the strict boundary between these disciplines, so clearly delineated in the past, has become blurred in recent years. In the last half century, biology has branched into numerous subdisciplines, many of which rely heavily on mathematics and physical and computer sciences. For example, a student of modern neuroscience is heavily immersed in the principles of physics and is well advised to acquire computational expertise. Increasing numbers of universities now offer degree programs in the fields of theoretical and computational biology. Despite some notable exceptions (26), computational and experimental studies in immunology have largely followed parallel tracks. This is perhaps partly due to an almost stereotypical "math phobia" that afflicts many biologists (including immunologists) but is largely because, until now, the value and benefits of such a marriage may not have been immediately obvious. Images of collective dynamic phenomena and the difficulties associated with intuitively discriminating between the consequences of different mechanistic hypotheses regarding the cooperative events is changing this situation rapidly.

Inherent with change is the need to adapt. The invention of the telescope gave us the ability to see what could never have been seen before. Out of this capability arose the need for ways to extract, interpret, and exploit the fruits of this powerful new technology of visualization. The result was classical mechanics (24). In a very literal sense, biology today is in a similar situation, where new imaging technologies are revealing "worlds" that have never been seen before, from the in vivo setting to the subcellular. The development of methods to quantitate visual data in immunology and understand it at a mechanistic level will allow us to take steps toward developing predictive algorithms and protocols for intervention when things go awry. Achieving this lofty goal will require a multidisciplinary approach, involving the fields of biology, mathematics, physics, chemistry, and computer science. An unprecedented level of outreach by biologists, physical scientists, and engineers is required to forge such collaborations. A blending of disciplines has not been business as usual in mainstream immunology, but such synergies appear to be necessary if we are to move beyond the stunning images and reap the full benefits of advanced imaging technology.

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