KINETIC MODELLING APPROACHES TO *IN VIVO* IMAGING

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The ability to visualize protein dynamics and biological processes by *in vivo* microscopy is revolutionizing many areas of biology. These methods generate large, kinetically complex data sets, which often cannot be intuitively interpreted. The combination of dynamic imaging and computational modelling is emerging as a powerful tool for the quantitation of biophysical properties of molecules and processes. The new discipline of computational cell biology will be essential in uncovering the pathways, mechanisms and controls of biological processes and systems as they occur *in vivo*.

> Science is built up of facts just as a house is built up of stones, but a collection of facts is no more a science than a heap of stones is a house.
>
> *H. Poincaré*

Ever since the first microscope was built in the seventeenth century, morphological observations by microscopy have driven the course of biology. Microscopes allowed the discovery of the cell as the structural unit of tissues and organisms, and they provided the tools to uncover the interior structure and organization of cells. Later, fluorescence-labelling methods with antibodies allowed the visualization of specific proteins within cells, and the development of microscopy systems with increasingly better spatial and temporal resolution — such as CONFOCAL and MULTI-PHOTON MICROSCOPY — has resulted in a detailed description of cellular architecture. Despite their success, conventional microscopy methods suffer serious limitations. They require chemical fixation and involve the observation of biological samples under non-physiological conditions. Conventional microscopy methods can generally not resolve the dynamics of cellular processes and, most importantly, it has been very difficult to generate quantitative data using conventional microscopy.

The discovery of genetically encoded fluorescent tags has revolutionized the way microscopy is used in biology. It is now possible to analyse the dynamics of proteins or organelles in living cells[1] and to probe interactions between molecules *in vivo*[2,3]. More relevant to the topic of this discussion, by combining *in vivo* microscopy with computational approaches, it is now possible, for the first time, to extract quantitative information about the biophysical properties of proteins within living cells. We discuss here some of the kinetic microscopy methods that provide the basis of quantitative *in vivo* imaging and we outline computational approaches to extract biophysical information from *in vivo* imaging data.

CONFOCAL MICROSCOPY
A microscopy method used to obtain a thin optical section through a specimen.

MULTI-PHOTON MICROSCOPY
A microscopy method that uses the simultaneous absorbance of several low-energy electrons to generate an optical section through a specimen.

FLUOROPHORE
A small molecule or a part of a larger molecule that can be excited by light to emit fluorescence.

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Kinetic microscopy

Recent advances in microscopy methods have made it possible to visualize the dynamics of proteins and organelles in living cells. To do so, proteins can either be covalently labelled with a FLUOROPHORE and injected into cells, or fluorescent tags can be genetically encoded[1,4–6]. By far the most popular fluorescent label is the autofluorescent green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. GFP can be fused to any complementary DNA using standard cloning methods, and, as several spectrally distinct derivatives are available, multicolour observations are possible on several proteins simultaneously[7,8]. An alternative genetically encoded tag is the FLASH system (fluorescein arsenic helix binder), which consists of a short peptide that is engineered onto a protein of interest[9]. The protein is then expressed and the cells treated with a peptide-binding ligand, which diffuses into the cell and fluoresces once it is bound to the tag. This system has not been widely used, but is

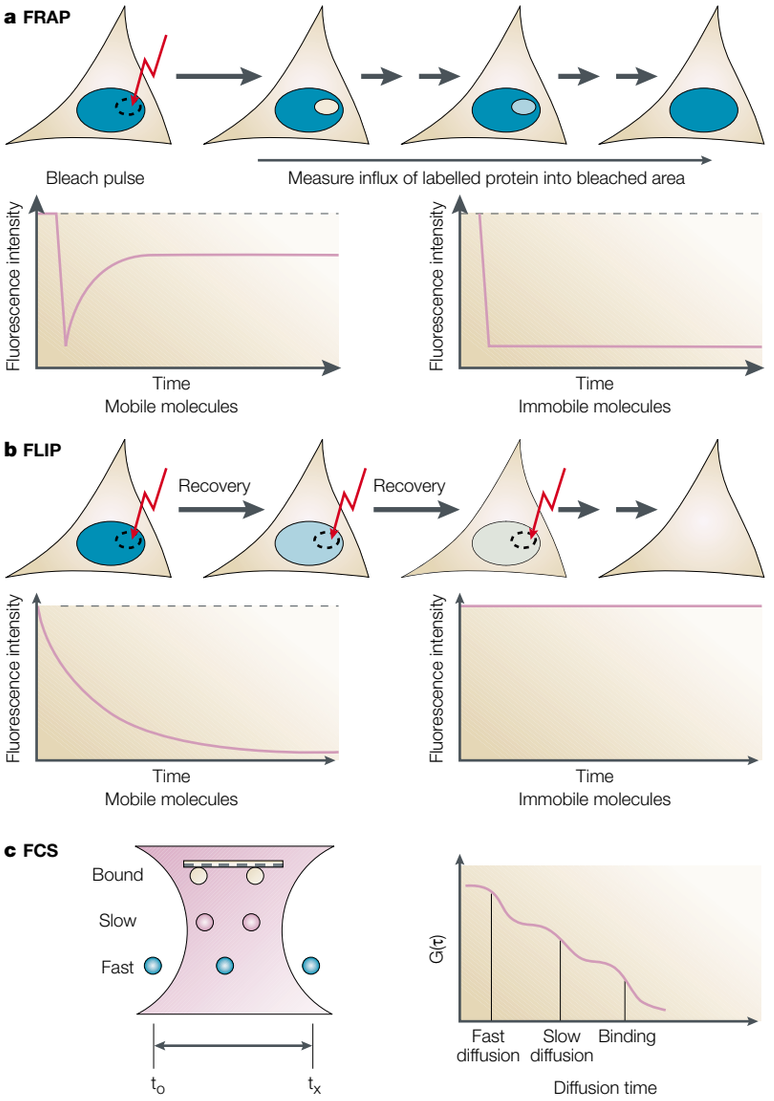


Figure 1 | **Kinetic microscopy methods. a** | In fluorescence recovery after photobleaching (FRAP), the fluorescence intensity in a small area after bleaching by a short laser pulse is measured using time-lapse microscopy. The recovery kinetics of mobile molecules are dependent on the mobility of a protein. Immobile proteins show no recovery. **b** | In fluorescence loss in photobleaching (FLIP), the fluorescence intensity is measured in a small area after repeated bleaching of a region that is distant from this area. The rate of loss of fluorescence signal is dependent on the mobility of the protein. Immobile proteins show no loss. **c** | In fluorescence correlation spectroscopy (FCS), the movement of molecules through a small volume is observed. During an observation period (t_x) rapidly moving molecules traverse the sample volume, whereas slowly moving molecules or bound molecules remain in the sample volume. Fractions of a protein with distinct mobilities are resolved by autocorrelation curves.

STEADY STATE
An open system, the content of which is held constant by a continuous input. Here, the output equals the input.

DIFFUSION COEFFICIENT
A measure to characterize the speed with which a particular molecule moves in a particular medium when driven by random thermal agitation.

attractive, as the small tag is unlikely to interfere with the function of the protein.

Genetically encoded tags are routinely used in time-lapse microscopy experiments to probe the dynamic behaviour of proteins and cellular compartments. For example, tracking the progress of GFP-fusion proteins through the exocytic and endocytic pathway has provided fundamentally new insights into protein transport and the architecture of the endomembrane system[10–12]. Similarly, time-lapse observations of the nuclear envelope[13,14], intranuclear structures[15–18], and genes and chromatin[19–23] have given the first insights into the dynamics of the cell nucleus[19–23]. Whereas many of the early *in vivo* microscopy studies were limited to observations of single focal planes at each time point, recently developed rapid sampling methods combined with the increased stability of fluorescent probes, now make it possible to routinely acquire three-dimensional data sets over time — a method commonly referred to as four-dimensional (4D) microscopy[24,25]. These types of experiments generate large data sets, which often cannot be intuitively and quantitatively interpreted. Visualization tools that facilitate the qualitative and quantitative analysis of 4D-data sets have recently been reported[26–28].

F words of kinetic microscopy: FRAP, FLIP, FCS

FRAP. Observations from studies with fluorescently tagged proteins typically show the STEADY-STATE distribution of a protein, but they do not directly provide information about the kinetic properties of molecules. To determine the kinetic properties of proteins *in vivo*, the movement of the protein of interest must be made visible. The most commonly used technique for this is FRAP (fluorescence recovery after photobleaching)[1,29–31] (FIG. 1). In this method, a small area of a cell is rapidly bleached using a high-intensity laser pulse. The movement of unbleached molecules from the neighbouring areas is then recorded by time-lapse microscopy as the recovery of fluorescence in the bleached area. This method is minimally invasive and the dynamics of the observed protein closely reflect its behaviour *in vivo* as FRAP does not generate protein gradients, but merely makes a fraction of the fluorescently labelled molecules invisible. FRAP experiments often give a qualitative impression of the mobility of a protein, but more importantly, they contain much quantitative information. For example, the apparent DIFFUSION COEFFICIENT and the size of the mobile fraction of a protein can be directly determined from the primary data[1,31].

FLIP. A related method to FRAP is FLIP (fluorescence loss in photobleaching). In this technique, an area within the cell is repeatedly bleached and the loss of fluorescence in areas that are distant from the bleach area is monitored[1] (FIG. 1). FLIP largely eliminates the concern that the recovery properties are due to damage at the bleach spot, as all measurements are made in areas that are never bleached. FLIP is often used to probe the mobility of a protein, but is also a useful tool to study the continuity of cellular compartments[32].

FCS. FCS (fluorescence correlation spectroscopy) provides an alternative method for measurements of protein dynamics *in vivo*[33,34] (FIG. 1). In FCS, a laser beam is focused on a microvolume, typically in the femtolitre range, and fluctuation of the fluorescence signal is measured over a short period of time. The recorded signals reflect the movement of labelled proteins through the sample volume, which is similar to the situation if we looked straight across a busy road and record the fluctu-

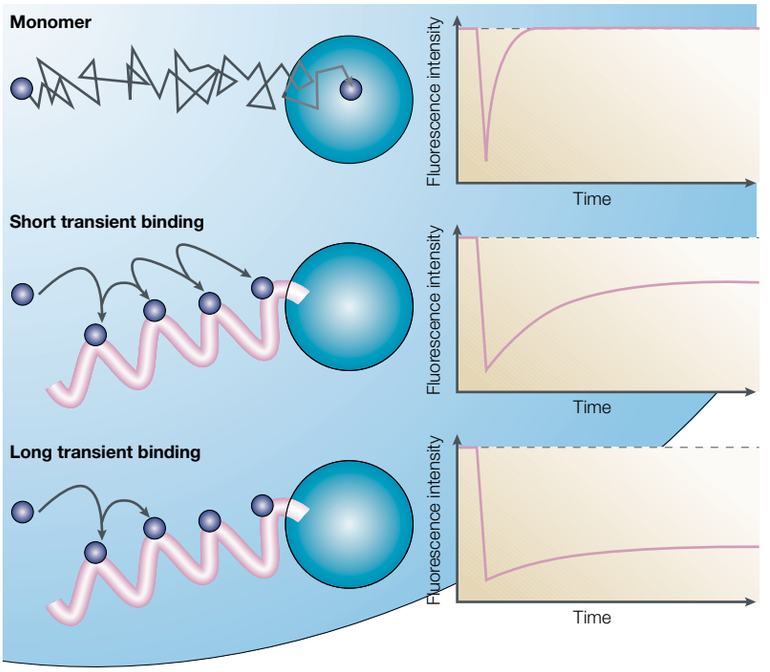


Figure 2 | **FRAP for the measurement of protein binding *in vivo*.** Information about the binding properties of a protein can be obtained from measurements of apparent protein mobility by FRAP. A monomeric protein moves more rapidly than a protein that transiently interacts with relatively more immobile cellular structures, such as the cytoskeleton or chromatin. The FRAP recovery kinetics reflect these transient interactions. A monomeric protein recovers rapidly, a protein with short transient interactions recovers with intermediate kinetics and a protein with long transient interactions recovers slowly. Kinetic modelling allows us to extract information about the binding properties from mobility measurements.

PARAMETER
The numerical constant that determines the absolute speed of a process. A first-order process is characterized by a single parameter, the rate constant. A process that is governed by a Michaelis–Menten equation is characterized by two parameters, V_{max} and K_m.

STOCHASTIC SYSTEMS
A dynamic system, the processes of which are characterized by a probability distribution. The stochastic system theory is particularly important when the abundance of molecules in a particular state falls below the deterministic limit, about 100 molecules per cell.

FRACTALS
These are objects that provide more and more features as the resolution of the observation increases. These finer features show statistical self-similarity as seen in biological branching patterns, ion-channel currents and heart rate.

ations in the passing cars. The time it takes molecules to move through the sample volume depends on their diffusional properties, and the fluctuation patterns give a direct measure of the concentrations of the proteins in the sample volume. FCS can be used to measure diffusion coefficients and binding constants[35–37]. As FCS can simultaneously be done on several fluorophores, it can also be used in cross-correlation spectroscopy to determine whether two proteins physically interact — if they do, they would be predicted to show identical fluctuation patterns. FCS is a single-molecule detection method and it is therefore exquisitely sensitive. However, owing to its great sensitivity, bleaching effects during measurements can generate artefacts. FCS is still in an experimental stage, but it does hold great promise. Early FCS applications include the determination of diffusional mobilities of proteins and RNA[35,36,38], the determination of binding interactions[39,40] and the measurement of ion concentrations[40].

Measuring biophysical properties *in vivo*

Photobleaching methods were initially designed, and are commonly used, to measure the mobility of proteins in living cells. Indeed, in FRAP and FCS, the primary measurement is the diffusion coefficient. However, as all proteins readily interact with many partners *in vivo*, it is virtually impossible to measure pure diffusional mobility of a protein in a living cell (FIG. 2). Furthermore, proteins often form higher-order complexes or associate with relatively immobile cellular structures, such as the cytoskeleton or chromatin. In both cases, the measured mobility is significantly reduced. It is important to realize that all photobleaching methods measure apparent mobilities[41]. This behaviour becomes obvious in the analysis of nuclear proteins. For example, the core histone H3 is virtually immobile, whereas a similarly sized splicing factor is highly mobile[42,43]. A similar situation is found in the analysis of plasma membrane and transmembrane proteins in membranous compartments[32,44,45].

The fact that the interaction properties of a protein are reflected in the mobility measurement complicates the analysis of photobleaching data (FIG. 2). However, the fact that binding to other cellular components affects mobility also indicates that the measured apparent mobility contains information about the behaviour of the protein. Computational approaches, particularly kinetic modelling methods, allow the quantitative testing of hypotheses about why a protein moves slower than would be expected for its size. The strategy in such an approach is to generate a mathematical description — a kinetic model — of the hypothesized biological reality. The model is characterized by biophysical PARAMETERS, such as binding and release constants, residence times and diffusion coefficients. By determining the set of parameters that result in a best fit of the model to the experimental data, the model can be tested and quantitative information about its parameters can be obtained. We outline below step-by-step how, using computational methods, we can extract information regarding biophysical properties of proteins and processes from *in vivo* microscopy data.

Modelling = quantitative hypothesis testing

Techniques for the analysis of dynamic data can usefully be divided into four groups, although the distinctions are not absolute: the statistical tools of time series analysis[46], the analytical and computational tools of differential equations[47–51], the computational tools of STOCHASTIC SYSTEMS[52,53], and the scaling and phase-space tools of FRACTALS and CHAOS[54]. For applications in cell biology, differential equations are often used because they are a natural mathematical language for cellular processes, such as biochemical kinetics, membrane transport and binding events. Alternative approaches to biological modelling and data analysis are described briefly in BOX 1.

The object of any type of data analysis is hypothesis testing. For example, statistical tests, such as the t-test or ANALYSIS OF VARIANCE, ask how probable it is that a particular experimental result occurs by chance. Analysis of quantitative dynamic data, such as those collected in video microscopy, goes one step further — it offers the possibility of quantitative mechanistic hypothesis testing. One way to visualize the role of kinetic modelling in cellular and molecular hypothesis testing is shown in FIG. 3. The difficulty is knowing with precision what is predicted by a complex

Box 1 | **Other types of mathematical models**

There are many approaches to mathematical modelling. Several other types of mathematical descriptions, apart from the dynamic, differential-equation models that are the focus of this review, are compared below.

Curve fitting.
Modern graphics and spreadsheet software typically have various options for fitting experimental data to functions. These tools offer a powerful means of summarizing a data set by fitting it to a SUM OF EXPONENTIALS, a POLYNOMIAL, a SUM OF GAUSSIANS, etc. This is always possible and the coefficients that fit the data can be taken as quantitative parameters, which are derived from the data. Indeed, these parameters are sometimes touted as model independent and therefore desirable. In general, however, these coefficients have no physical meaning. They can quantify patterns and can be useful in diagnosis, but they yield no mechanistic information. By contrast, the parameters of a mechanistic model yield useful information on the biological processes that they quantify. If, for example, the rate constant for a given process is doubled in a given experimental circumstance, then the corresponding process has been fundamentally changed by the experimental protocol.

Statistics.
Statistical models search for patterns in experimental data. Correlation, regression and cluster analysis are all powerful statistical tools that can identify relationships among measured variables that probably are not attributable to chance. Statistics are a powerful tool for supplying us with new and interesting potential mechanisms, mechanisms that need experimental tests and mechanistic analysis.

Phenomenological laws.
Many readers will be familiar with linear phenomenological laws, such as Ohm's law for electric current ($I = g\Delta V$) or the law of bulk flow ($F = (1/R)\Delta P$). These seem to be algebraic, but are really special cases of the general linear differential equations that govern forces and fluxes in irreversible thermodynamics[64]. The current (I) is the rate of charge movement, dQ/dt; and the flow (F) is the rate of volume movement, dV/dt. The 'forces' in these equations (ΔV and ΔP) are gradients in chemical potential and can, when required, be resolved into functions of the state variables whose derivatives are on the left-hand sides of the differential equations.

Enzyme kinetics.
The often-cited form of the Michaelis–Menten equation, $v = V_{max}S/(K_m + S)$, can be taken as a surrogate for all the more complex velocity equations that are derived and documented in REF. 65 and elsewhere. In the context of differential equation models, we can incorporate enzyme kinetics by recognizing that this equation is another differential equation in disguise. Velocity (v) is a symbol for the rate of change of product, dP/dt. This most widely known of biochemical equations is therefore a nonlinear differential equation in which the driving force of chemical potential arises from a concentration of substrate (S), which is greater than its equilibrium value.

Logic models.
An array of modelling tools, which have been developed in theoretical biology, systems biology and biomedical engineering, are beginning to move from qualitative to quantitative simulations. These include electric circuit models[49], logic network models, Petri nets and process algebra.

CHAOS
A deterministic system (for example, some systems of nonlinear differential equations), the output of which seems random, but is not. Such systems show a surprising sensitivity to initial conditions.

ANALYSIS OF VARIANCE
A statistical procedure for testing for differences among the means of several populations. It partitions the total sample variance among several specific sources to carry out the test on means.

SUM OF EXPONENTIALS
An algebraic expression that is made up of exponentials. In a first-order system, the time-course solution for every state can be precisely mimicked by the sum of exponentials that correspond to the number of states in the system.

POLYNOMIAL
Algebraic expressions that are made up of more than one term — for example, mx + b.

SUM OF GAUSSIANS
An approximation by weighted sums of normal distributions, or Gaussians, each characterized by two parameters — a mean and a variance — to describe a data set.

STATE
The generic name used here to identify those variables that change with time and for which differential equations are written.

hypothesis that contains many interacting processes in a given experimental situation. It is here that kinetic modelling and simulation of complex systems have proved valuable.

Molecular and cell-biological hypotheses or theories are often represented as diagrams. The final slide in nearly every scientific seminar and a key figure in every biological review is a diagram that represents the author's current working hypothesis. Typically, these diagrams comprise a collection of molecules represented as symbols, their biochemical transformations and translocations represented as arrows, and some graphic notation, such as lines that are terminated with plus or minus, to indicate regulatory controls. Diagrams offer a language for description of cell-biological systems that is somewhat more precise than printed paragraphs, but it often remains difficult to make quantitative predictions for a given experimental protocol with the use of diagrams alone. Scientific intuition has been successful when systems are limited to a few molecules and processes, but today's summary diagrams generally have many more molecules and arrows than this, and even simple systems often behave in surprising ways. What is needed is a way to know what the diagram predicts in a given experiment.

Translating diagrams to kinetic models

Kinetic modelling supports quantitative hypothesis testing by first translating a diagram into a mechanistic kinetic model (see BOX 2). Diagrams typically consist of molecules, complexes, cellular locations and processes. As molecules and complexes can exist in several locations, it is often necessary to define several STATES for a single molecule — each state is 'a chemical species in a physical place.' For example, phosphorylation of a protein at a single amino acid results in at least two states: the phosphorylated one and the unphosphorylated one. However, if both are present in both cytoplasm and nucleoplasm, then four states are required.

Arrows in a diagram usually represent various types of processes. Three fundamental biological processes can describe virtually any biological diagram: transformations, translocations and binding.

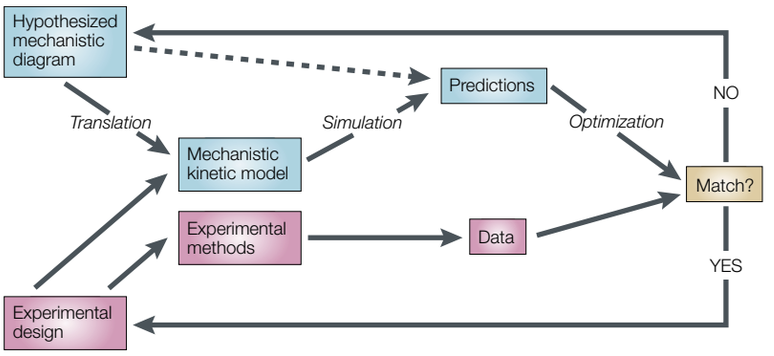


Figure 3 | **Role of kinetic models in quantitative hypothesis testing.** The lower (purple) half of this figure represents experimental design and experimental methods, which combine to produce quantitative experimental data on a biological system of interest. Hypothesis testing is then carried out by comparison of the data to that expected based on the current hypothesis. Arrows in the upper (blue) half of the diagram represent the steps in kinetic analysis that are detailed in this review: translation of diagrams into kinetic models, simulation to obtain the predictions of the model for a given experimental design and optimization of model parameters to ensure an unbiased comparison of model predictions and experimental data. These three steps differ from scientific intuition (dashed arrow) by being quantitative and robust to complexity.

Transformations include everything that makes or breaks covalent bonds, such as the biochemical pathways of intermediary metabolism, the synthesis and splicing of RNA transcripts, the actions of kinases and phosphatases, and the proteolytic activities of proteasomes or caspases. Translocations include all active and facilitated transport mechanisms, currents in ion channels, diffusion and bulk-flow processes that move molecules or complexes from one place to another. Binding comprises all those intermolecular interactions, such as hormones that activate receptors, ALLOSTERIC REGULATION of enzymes or transporters and formation of multimeric protein complexes, that involve bonds whose energy is much less than the energy of a covalent bond.

A biological diagram is therefore a collection of processes that link states. To express the diagram in mathematical form requires two steps. First, a RATE LAW is written for each PROCESS, and second, these rate laws are combined to construct the differential equations for each state. A rate law is an algebraic expression that gives the flux (molecules per second) through a particular process as a function of the relevant molecular abundances or other state variables in the biological system. Rate laws tell us how many molecules are traversing a particular pathway, but to keep track of how many molecules there are in any state at any time, it is necessary to sum up or integrate all the inputs and outputs. To do this, differential equations are constructed for each state (molecule or molecular complex in a cellular location) by setting its rate of change to be equal to the sum of the processes or rate laws that produce this molecular species minus those processes or rate laws that consume it. In other words, we write down the differential equations that represent mass conservation for each state[55]. BOX 2 gives an example of the diagram translation process.

Because most biological diagrams concern themselves with changes in more than a single molecular species, the corresponding kinetic models comprise 'systems' of differential equations. For example, if the diagram contains 17 molecular species, the corresponding model will, in general, consist of 17 differential equations. The form of these equations is perhaps most easily understood by examining the dimensions or units of the various terms. Traditionally, the derivatives are written on the left-hand side of these equations. These derivatives with respect to time represent the instantaneous change in molecular abundance, or the rate of change of the number of molecules of this species in a particular cellular location at a particular moment in time, measured in, for example, molecules per second. As the units on the right-hand side must be the same as those on the left, it is clear that the derivatives must have the same units as the fluxes. Processes or fluxes that produce or deliver the molecular species will have a positive sign; those that consume it or remove it will have a negative one.

By writing rate laws, diagrams are translated into a precise physical–chemical language. The reason for using differential equations rather than algebraic equations is that differential equations are able to describe mechanisms and cause-and-effect relationships. The change on the left-hand side of a differential equation (the derivative) can be seen as the effect, and the processes on the right-hand side can be seen as the causes. The complete differential equation therefore expresses the equality of cause and effect.

The rendering of biological diagrams in mathematical form does not mean that cell biologists must know how to solve differential equations using analytical, pencil-and-paper methods. Indeed, for many nonlinear differential equations, no such solution is even possible. Fortunately, there are many software tools (see online links box) available to solve any system of differential equations, from very simple to very complex. Although the typeset form of a system of differential equations is fairly standardized, it is remarkable how many differential equation-solver software packages with different input formats are currently available. Attempts to construct an 'Esperanto' for biological kinetic models are under way, and are based on the popular EXTENSIBLE MARKUP LANGUAGE (XML). This means that a model file not only encodes the model-definition information, but also encodes how to interpret it. This new language could substantially facilitate the communication of models between software packages and among laboratories. Further details are available in the published descriptions of CellML[56] and SBML[57], as well as in the links included at the end of this review.

Simulation: what does a diagram predict?

Once a biological diagram has been formulated as a quantitative kinetic model, simulation is used to discover what that model predicts for a particular experimental situation. In other words, simulation applies a particular experimental protocol to the kinetic model and displays the model's predictions in a way that can be compared directly to the experimental data (FIG. 3).

ALLOSTERIC REGULATION
A modification of a process by a molecule that binds to an enzyme or a transporter or another protein at a site other than its active, or catalytic, site.

RATE LAWS
Algebraic expressions for the flux through a given pathway.

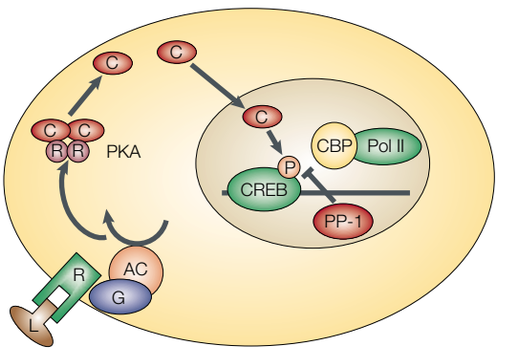
PROCESS
The generic name for events that bring about changes in one or more states.

EXTENSIBLE MARKUP LANGUAGE
A method for putting structured data in a text file so that applications receive not only unambiguous data but also unambiguous context. XML documents are not meant to be read, except by software.

Simulation provides answers to key questions that cannot be answered with confidence in any other way. You can discover whether a diagram really is consistent with experimental data. You can also learn whether a diagram is consistent with experiments that have been reported by other laboratories and, just as importantly, you can carry out the same tests for diagrams that have been proposed by other investigators. Simulation requires a quantitative language for laboratory procedures so that these can be imposed on the kinetic model just as they are imposed on the cells in the laboratory. Some modelling software packages (for example, SAAM II) have an experiment toolbox to facilitate quantifying your protocol and defining your measurements. Others (for example, Berkeley Madonna, MATLAB, Virtual Cell) provide a flexible set of programming constructs that can be used to specify the sequence of events that defines a protocol.

A protocol is a timeline of pre-planned experimental perturbations. Nearly always, it consists of experimenter-induced changes in state variables or processes that are taking place at known times. In practice, this translates to specifying a set of initial and boundary conditions to be imposed on the solution of the differential equations. For example, FRAP experiments are often assumed to begin in a steady state. This means that the steady-state solution of the differential-equation system can be calculated and used to set the initial condition for each molecular abundance before initiating the bleach pulse. The same applies to other types of experiments. If a growth factor or a hormone is added to the medium, then the corresponding state variable is increased stepwise at the appropriate time.

Box 2 | **Translation**



A diagram[66] that contains all three main classes of biological processes — transformation, translocation and binding — is reproduced here to illustrate the translation process. When a ligand (L) binds to its receptor (RGAC), adenylyl cyclase is activated and cyclic AMP (not shown) is synthesized from ATP. The first differential equation represents binding. The derivative (*dLRGAC/dt*) on the left-hand side represents the rate of change of ligand–receptor–G-protein–cyclase complex, and the terms on the right-hand side represent the binding and release of ligand. The second equation represents the rate of change of cAMP, and the two processes are both transformations — the first term represents the flux (molecules per second) of cAMP that is produced by adenylyl cyclase and the second represents the flux of cAMP degradation by phosphodiesterase. Either or both of these simple enzymatic rate laws could be replaced with more complex ones if, for example, saturation or allosteric regulation of the enzymes was thought to be important.

$$dLRGAC/dt = k_f \cdot L \cdot RGAC - k_r \cdot LRGAC$$
$$dcAMP/dt = k_{AC} \cdot LRGAC \cdot ATP - k_{PDE} \cdot cAMP$$

The mechanism of control of protein kinase A (PKA) is summarized in the diagram without kinetic detail both because it is well known and because it is not the focus of the review. For our purposes, the catalytic subunit of PKA can be considered to exist free or bound to its regulatory subunit.

$$dCR/dt = k_{bind} \cdot R \cdot C - k_{unbind} \cdot CR$$
$$dR/dt = k_{unbind} \cdot CR - k_{bind} \cdot R \cdot C - k_{cAMP} \cdot R \cdot cAMP + k_1 \cdot RcAMP$$
$$dC/dt = k_{unbind} \cdot CR - k_{bind} \cdot R \cdot C - k_{InNuc} \cdot C + k_{OutNuc} \cdot C_{Nuc}$$

If the abundance of R is very small compared to cAMP, then the last two terms in *dR/dt* can safely be omitted from the right-hand side of *dcAMP/dt*. Otherwise, we must add these terms. The equation for *dC/dt* emphasizes that C can exist in two places, and therefore includes the first translocation processes in this example. Note that the simple first-order rate laws for nuclear import and export could readily be replaced by more mechanistically detailed ones if desired.

$$dC_{Nuc}/dt = k_{InNuc} \cdot C - k_{OutNuc} \cdot C_{Nuc}$$
$$dCREB/dt = -k_{PKA} \cdot C_{Nuc} \cdot CREB + k_{PP-1} \cdot PP1 \cdot CREBP$$

Once in the nucleus, the catalytic subunit mediates phosphorylation of the cAMP response element binding protein (CREB). Then, CREBP recruits the CREB-binding protein (CBP), which, in turn, recruits RNA polymerase II. Recruitment is easily modelled as binding and the transcription rate can be seen as proportional to the abundance of this heteromeric complex.

$$dCREBP/dt = k_{PKA} \cdot C_{Nuc} \cdot CREB - k_{PP-1} \cdot PP1 \cdot CREBP - k_{recruit} \cdot CREBP \cdot CBPPPolII + k_{dis} \cdot CREBPCBPPolII$$
$$dCREBPCBPPolII/dt = k_{recruit} \cdot CREBP \cdot CBPPolII - k_{dis} \cdot CREBPCBPPolII$$
$$Transcription\ Rate = k_{elong} \cdot CERBPCBPPolII$$

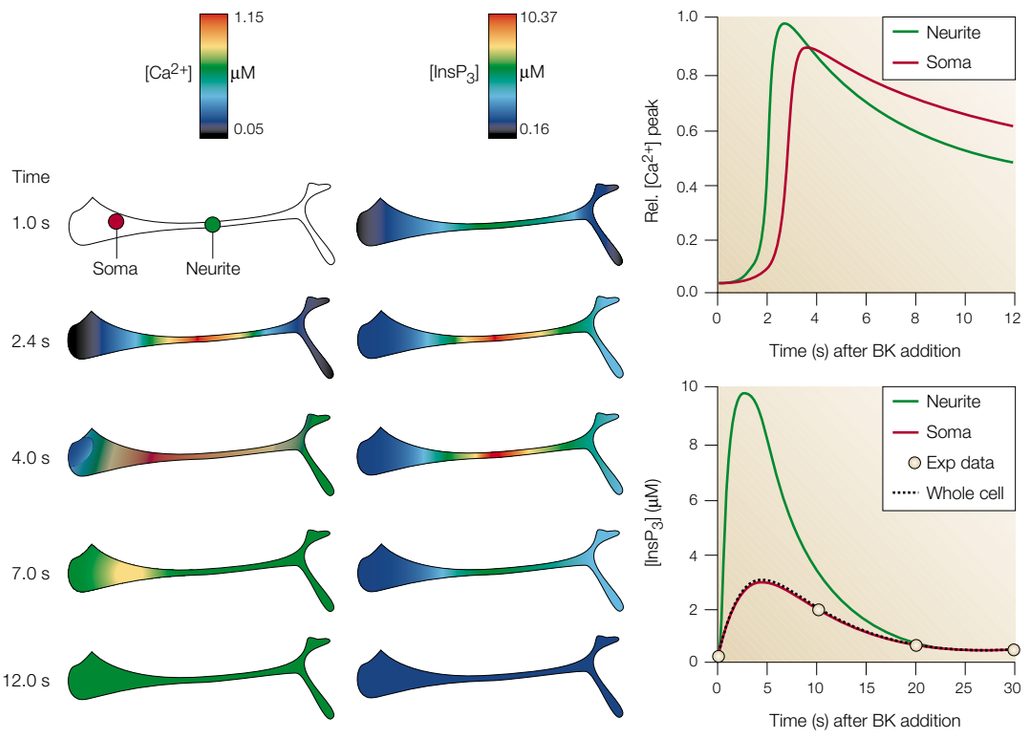
And if an enzyme inhibitor or receptor antagonist is added, a specific rate constant could be altered at the right time to initiate the response of the model. Imposing these boundary conditions on a kinetic model leads to the definition of the boundary of a model as those molecular species or physical quantities that impinge on the model, but for which no differential equation is written. Variables on the boundary must be completely specified in the protocol because they affect the outcome but are not explicitly modelled.

FRAP protocols are initiated by defining a region of cellular space to be bleached and then changing a bleaching rate constant to a non-zero value for the duration of the experimental laser pulse. FLIP experiments are imposed on a model by bleaching as is the case for FRAP, but with an average effective bleach constant, or by a more precise replication of the repeated laser pulses with intervening periods of image collection.

Simulation can also be used to show what a particular biological diagram predicts for every defined region

Box 3 | **Simulation**

When both time and space are variables, mathematicians refer to the resulting differential equations as partial differential equations. To solve these, the space is partitioned into a large number of small, but finite, volumes. This amounts to compartmentalizing the space and then solving ordinary (only time is an independent variable) differential equations for every volume simultaneously. Shown here is such a simulation for Ca^{2+} and inositol-1,4,5-trisphosphate ($Ins(1,4,5)P_3$) dynamics in a neuroblastoma cell[59]. Note that the computing times are vastly different for partial differential equations versus ordinary differential equations. These simulations each required about 25 minutes on an SGI workstation; if diffusion could have been neglected and the Ca^{2+} and $Ins(1,4,5)P_3$ compartments treated as well mixed, these simulations could reasonably have been done in a few seconds. The pseudocolour images graphically depict the evolving solutions of this model for Ca^{2+} and $Ins(1,4,5)P_3$ at more than 7,000 locations, each 1.2 μm on a side, in the simulated neuroblastoma cell. Graphs at the right of this figure show the simulated time courses in two selected locations, one in the soma (red) and one in the neurite (green). The original paper and the online supplementary material should be consulted for details of model structure and equations, but it is instructive to realize that this kinetic model makes predictions for a system with at least ten interacting processes: (1) dynamics of $Ins(1,4,5)P_3$ synthesis, (2) the spatial distribution of bradykinin (BK) receptors on the cell surface, (3) the spatial distribution of SERCA (SR–ER calcium) pumps, (4) $Ins(1,4,5)P_3$ receptors and (5) leak channels in the endoplasmic reticulum, (6) the spatial distribution of Ca^{2+} channels and (7) pumps in the plasma membrane, and Ca^{2+} buffering by both (8) fixed endogenous buffers and (9) mobile exogenous ones (Fura-2 in this case), as well as (10) diffusion of the mobile species. Applications such as this one illustrate the profound usefulness of simulation as the best tool for discovering what your diagram predicts. Figure courtesy of L. Loew.



of a spatially complex cell. This is essential whenever diffusion cannot be assumed to be fast on the timescale of the experimental measurements, as is often the case for FRAP experiments, or for experiments in large cells or nerve cells with long projections. One of the most interesting tools for such 'four-dimensional' simulations is the Virtual Cell software[58]; an excellent example of its use in simulating whole-cell calcium dynamics imaged with a fluorescent dye (Fura-2) has recently been published[59] (see BOX 3 for details).

Simulation versus modelling

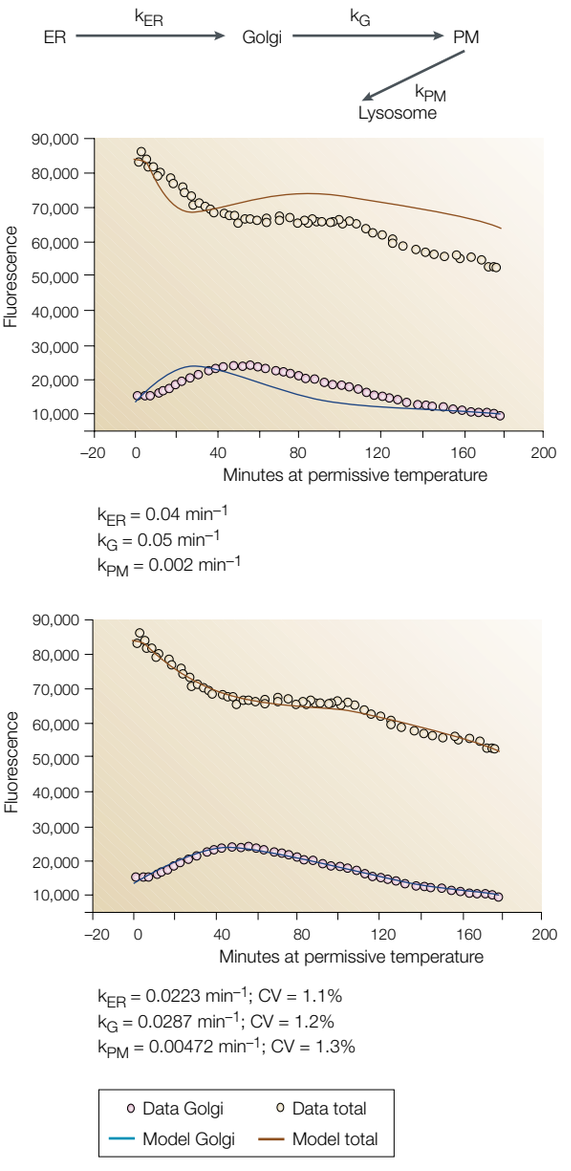
The terms 'simulation' and 'modelling' are often used interchangeably, but it is useful to make a distinction. Simulation typically makes no explicit reference to experimental data, whereas modelling generally links each experimental measurement to some function of the state variables of the model and then assesses the model's ability to reproduce the data when the corresponding experimental protocol is applied.

Simulation is therefore the first step in modelling. Once a model of the biological system has been proposed and a model of the experiment has been superimposed on it, simulation permits us to know with precision what the model predicts. But in practice we need to know precisely how to decide whether a model fits the data or not. How do we find the best combination of parameters? The answer is optimization. Optimization theory[60] is therefore an essential element of kinetic analysis and modelling (FIG. 3).

Box 4 | **Optimization**

The figure shows the classic model of the secretory pathway with proteins transported from the endoplasmic reticulum to the Golgi complex and to the plasma membrane. Data points in the graphs represent the transport of VSVG–GFP (vesicular stomatitis virus G protein–GFP) through the Golgi apparatus in a single cell[61]. Solid lines in the upper panel represent a solution of the secretory pathway model for the values of the rate constants below the graph. Clearly, this model fails to fit the experimental data for total cellular fluorescence (yellow) and Golgi fluorescence (pink). We would not, however, want to rule out the classical ER–Golgi–plasma-membrane model on these grounds because the poor fit is only a consequence of wrong parameter values. In the lower panel, the optimizer in the SAAM II software (see online links box) has minimized an appropriate objective function and found a point in parameter space that gives excellent fits (brown, blue)of the experimental data. Moreover, the coefficients of variation (CV) for the parameter estimates are all less than 2%, as shown below the graph.

This example emphasizes the usefulness of an optimizer in giving any theory its best chance to fit the available experimental data, as well as simultaneously supplying the very useful statistical information on the confidence that we should place in these parameter estimates. Again, it should be emphasized that these confidence limits hold for the given model structure. Residence times and fluxes that are calculated from these rate constants also hold for this structure and could well change if a new model, which fits the experimental data just as well, is discovered. Original data courtesy of K. Hirschberg, J. Lippincott-Schwartz).



INTEGRATIVE BIOINFORMATICS
The intersection of kinetic modelling and database technology, a combination that becomes essential as cell biologists move to analyse larger and more complex molecular genetic control systems.

Optimization

From the perspective of quantitative hypothesis testing, the purpose of optimization is to give each hypothesis its best chance to account for the available experimental data. Optimization is the exploration of the parameter space of a model in search of numerical values for each parameter that optimize a specific quantitative measure of goodness of fit. You can think of parameter space by imagining an origin with as many axes emerging from it as you have parameters in your model. Every point in this space represents a unique set of numerical values, one for each parameter. Now we add one more axis to this space, one that represents the measure of fit. One intuitively clear measure of fit is the weighted sum of the squares of the errors between the model solution and the experimental data. Every set of parameter values will correspond to a numerical value of the weighted sum of squares. For a perfect fit, the sum of squares would be zero. The task of optimization is to determine the set of parameter values that yields the best or optimal fit. For large models this is not easy, and improvement of methods for efficiently searching a high-dimensional parameter space represent an important research area in numerical analysis. BOX 4 gives an illustration of the optimization process that is applied to the kinetics of protein transport in the secretory pathway[61].

If a set of parameters has been found that fit the experimental data reasonably well, we can say that the theory is quantitatively consistent with the experimental data. This, in itself, is a stronger assertion than the paragraph of a discussion section that begins, 'Taken together, these data indicate…'. If the model is correct, then the parameter values of the mechanistic model can be used to calculate extra features of the system, including residence times, steady-state molecular abundance, steady-state fluxes (molecules per second) or fractional distribution of a given molecule among its various cellular locations. Moreover, if the parameters are evaluated in two or more physiological or pharmacological situations, it becomes possible to discern which cellular processes were affected and by how much. This is new information, much of it unavailable by any other means. All of these motivate the application of kinetic analysis to complex molecular and cellular problems.

Often, however, a model fails to account for all the experimental data. No set of parameter values can be found that eliminates systematic deviations between model solution and experimental data. This means either that the kinetic model is flawed and needs to be improved by ensuring that the biological diagram has been accurately translated, or that the biological diagram itself — that is, the theory — is incorrect. Computational tools are not yet used routinely to discover new theories, but this will probably become essential as complexity grows. A provocative example of a relatively simple computational search for an appropriate model recently appeared[62], and this general goal is a current objective of several research groups.

Optimization also provides tools to evaluate how well our analysis performs. Parameter estimation is able to provide confidence intervals for each parameter because the greater the noise in the experimental data, the greater is the uncertainty or variance that is associated with the optimized parameter values. The standard statistical measure of confidence in a parameter estimate is its coefficient of variation (CV). This number is calculated as the estimated parameter standard deviation divided by the parameter value, and is generally reported as a percentage. Some software packages report this information as the fractional standard deviation (FSD) of the parameter estimate, and other packages, even some that include optimizers, do not report this vital information at all. Another measure of confidence in a model is sensitivity analysis. In its most common form, this procedure involves solving the model again with one of its parameter values increased or decreased to show that this new value no longer fits the experimental data. Although qualitatively useful, this one-at-a-time variation fails to ask whether the fit, once destroyed by a change in the tested parameter, could be recovered by making changes in the other parameters of the model. For this reason, formal parameter estimation and its extensive statistical methods, which lead to coefficients of variation, are preferred.

Conclusions and prospects

Recent advances in applications of fluorescent tracers and indicators have permitted microscopy to move from static images to dynamic recording in live cells. The combination of these powerful imaging methods with mechanistic computational modelling allows us, for the first time, to extract information about biophysical properties of proteins and processes in living cells. Kinetic analysis and modelling aim to help investigators deal with cellular complexity by allowing them to know with precision what their complex diagrams predict. Quantitative predictions can then be compared directly with quantitative experimental data as a means of testing the hypotheses that are represented by the diagrams. Just as database tools have become vital to the field of bioinformatics for the management and statistical analysis of complex data sets, the management and analysis of large numbers of complex biological models is being facilitated by database technology and defines the nascent fields of INTEGRATIVE BIOINFORMATICS or pathway databases[63].

Biology knows, perhaps better than other scientific disciplines, the difficulty of analysing and understanding intact complex systems. It is not surprising that disciplines that routinely deal with complex systems, such as developmental biology or physiology, are strongly represented among the pioneers of computational biology. Kinetic analysis is now also beginning to find a place in the technical repertoire of cell biologists. In the coming years, computational cell biology and systems biology will be powerful tools to help us comprehend the enormous complexity of cell biology.

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DATABASES

The following terms in this article are linked online to:

Swiss-Prot: http://www.expasy.ch/
GFP

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Simulation and Modelling Software

SAAM Institute: http://www.saam.com/
Berkeley Madonna: http://www.berkeleymadonna.com/
Gepasi: http://www.gepasi.org/gepasi.html
StochSim: http://www.zoo.cam.ac.uk/comp-cell/StochSim.html
MATLAB: http://www.mathworks.com/index.shtml
Virtual Cell: http://www.nrcam.uchc.edu/
WinSAAM: http://www-saam.nci.nih.gov/
XPPAut: http://www.math.pitt.edu/~bard/xpp/xpp.html
E-Cell: http://www.e-cell.org/

Online Textbook

Integrative Bioinformatics: http://www.bioinformatics services.com/bis/resources/cybertext/IBcont.html

Databases

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KEGG: http://www.genome.ad.jp/kegg/
BIND: http://www.bind.ca/cgi-bin/bind/dataman
WIT: http://wit.mcs.anl.gov/WIT2/
EMP: http://emp.mcs.anl.gov/
EcoCyc:http://ecocyc.pangeasystems.com/ecocyc/ecocyc.html
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