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Article title: Modelling of Spatially-Restricted Intracellular Signalling

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Abstract

Understanding the signalling capabilities of a cell presents a major challenge, not only due to the number of molecules involved, but also because of the complex network connectivity of intracellular signalling. Recently, the proliferation of quantitative imaging techniques has led to the discovery of the vast spatial organization of intracellular signalling. Computational modelling has emerged as a powerful tool for understanding how inhomogeneous signalling originates and is maintained. This review covers the current imaging techniques used to obtain quantitative spatial data and the mathematical approaches used to model spatial cell biology. Modelling-derived hypothesis have been experimentally tested and the integration of modelling and imaging approaches has led to non- intuitive mechanistic insights.

A cell reliably responds to a myriad of extracellular signals by engaging cell surface receptors and modulating its extensive intracellular signalling machinery. This response may require the complex processing of inputs of varying amplitude or frequency. Some of these signals give rise to seemingly opposite physiological outcomes, despite their reliance on signalling networks sharing the same kinases, and transcription factors to propagate their message to a specific cellular output. How does the cell efficiently integrate these signals without compromising specificity?

An extensive body of experimental work has uncovered some of the exquisite temporal fine-tuning of network topology and kinetics required for these integrative processes to occur seamlessly. Only recently, due to advances in imaging techniques, has the spatial component of the regulation of cellular signalling been explored. This newly discovered contribution of sub-cellular localization, diffusivity and active transport adds another dimension of possible regulation to the integrative signalling response. The cell devises ways of creating inhomogeneous signalling, giving rise to microdomains, small subregions of concentrated molecules or active proteins. This may allow the cell to spatially segregate inputs and streamline processing, as part of its signal-handling repertoire. The inherent complexity of the intracellular signalling response requires a quantitative framework to gain insight into its organizational principles and possible emergent properties^{1, 2}.

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The aim of this review is to provide purely theoretical or experimental scientists a glimpse at the type of imaging and computational methods available to study spatial intracellular signaling. This review starts by briefly introducing some of the experimental techniques (along with their limitations) that are used to study spatial cell biology. These approaches provide measurements with the temporal and spatial resolution needed to develop computational models of intracellular signalling, and have been applied to uncover novel mechanistic insights into key signalling processes underlying cytokinesis and t-cell activation^{3, 4}. This review also addresses the existing theoretical approaches to study the possible origins of inhomogeneous signalling and explores the power of integrating theoretical and experimental methods to understand intracellular signal processing and signal specificity.

Intracellular biology across time and space

Traditionally, cellular biology has relied on static *in vitro* measurements of low temporal resolution with little to no spatial information. Measurements of enzymatic reactions required the homogenation of cells or tissue, to obtain the appropriate quantity of enzyme to reconstitute the enzymatic reaction in a test tube. Immunoblotting techniques necessitate the lysing of cells, and the destruction of intracellular organization. Immunostaining techniques provide spatial resolution but rely on cross-linking, and do not permit real time monitoring of spatial dynamics.

Advances in fluorescence imaging methods highlighted the significant sub cellular spatial organization present in eukaryotic cells. The discovery of green fluorescent protein (GFP), its cloning, and subsequent use as an *in vivo* fluorescent tag revolutionized the visualization of intracellular dynamics⁵⁻⁷. Genetically encoded tags are currently used to track protein location, function, motility and turnover in the intact cell. They are also used in studies of mechanisms of protein targeting, tracking promoter activity and as molecular timers⁸⁻¹⁰.

The fluorescence emission profile of GFP, its homologues and mutation-induced variants encompass the whole visible spectrum, with the color diversity of fluorescent proteins ranging from violet-shifted to the far-red range¹¹. Thus, it is now possible to express and easily detect as many as six different fluorescent proteins in a single cell, allowing the multiplexing of imaging of tagged-proteins¹². Mutation-induced variants have also been developed to be chromophore-sensitive to their surrounding environment, making them dynamic *in situ* reporters for pH, concentrations of ions, and redox changes¹³⁻¹⁶. Some of these variants have been shown to be photoactivatable fluorescent proteins, meaning that upon excitation with a defined wavelength, they convert from non-fluorescent to fluorescent or shift their emission spectra to a higher wavelength range¹⁷.

The ease of monitoring fluorescence over time made fluorescent -based techniques vital to follow *in situ* temporal and spatial dynamics of many diverse processes. These techniques allow the tracking of intracellular events in the microsecond timescale and in their intact environment, making them complementary to any modelling effort. The methods described can be classified as techniques that measure motility and techniques that measure activity

states. All the experimental techniques described below rely on the exogenous expression of reporter proteins. Thus it is important to take note of this issue when interpreting results.

Measuring Protein Motility in living cells

FRAP- Fluorescence recovery after photobleaching

FRAP provides information on both the diffusivity of a protein and the proportion of it in a motile vs. stationary state within a defined compartment¹⁸. This imaging technique takes advantage of a fluorescent -tagged protein of interest expressed in a live cell. Using a high-powered laser and a scanning confocal microscope, a small region of the cell is illuminated until all the fluorescence from the tagged molecules has irreversibly disappeared (i.e. photobleached, Fig 1(a)). The same sub-region is subsequently monitored as tagged molecules from neighbouring non-photobleached regions repopulate it, and a new steady state is reached (Fig 1(a))¹⁹. Two types of quantitative information can be readily obtained from FRAP experimental raw data: motility rate and the motile fraction. The motile fraction is the proportion of the protein that is the motile state (freely diffusing) vs. stationary state (bound or immobilized or restricted by barriers). The new steady state reached after photobleaching represents the mobile fraction (fig 1(b)). Even though the immobile fraction proteins are photobleached to the same extent as those in the mobile fraction, they fail to exchange with neighbouring regions. The immobile fraction may eventually turnover but at much slower timescales than those of the experiment. The motile fraction is calculated as the ratio of the initial fluorescence (before the photobleaching event) to the final fluorescence corrected by the minimal fluorescence (fluorescence just after bleaching). Plotting the recovery fluorescence as a function of time, and then analyzing this curve with a proposed reaction diffusion model allows the calculation of the motility rate, or diffusion rate²⁰. The proposed reaction diffusion model can be constructed in several modelling softwares such as VCell (vcell.org). To further confirm the extent of the motile fraction, a second photobleaching event should result in a fully recoverable fraction. Recently, the use of photo-activatable fluorescent proteins have modified the FRAP paradigm. Instead of photobleaching the fluorophore, a laser is used to photoactivate the fluorescent protein, and the new fluorescence emission is tracked^{17, 21}. Unlike traditional FRAP, this approach allows the repetitive probing of protein motility.

FCS- Fluorescence correlation spectroscopy

FCS is technique more recently implemented to measure single molecule dynamics and diffusivity¹⁸. FCS measures the fluctuation of fluorescence signal within a small volume in a cell, and given its sensitivity, even dynamics of low abundance proteins can be analysed (Fig 2). Using a confocal microscope laser to excite a small-geometrically defined volume (in the low femtoliter range), fluorescently tagged proteins are imaged at high temporal resolution as they diffuse in and out of the volume (Fig 2 (a)). These diffusion events are captured as spontaneous fluctuations in the fluorescence signal over time, and thus can be eventually converted into diffusion constants²².

The temporal autocorrelation of the fluorescence signal $F(t)$, gives rise to a normalized fluctuation autocorrelation function $G(\tau)$ (Fig 2(b)). To do this, the raw fluorescent intensity

trace is first divided into discrete time bins. The fluctuation about the average fluorescence F at specific time (t) is multiplied with that at a time delay τ later to calculate the autocorrelation function²³. Thus, this compares the similarity of the fluctuations of fluorescence intensity at time t , and at a later time ($t + \tau$). This is then normalized by the average fluorescence intensity over time. The autocorrelation function $G(\tau)$ is plotted against the log of the time delay τ , also known as the correlation time (Fig 2(b)). The measured autocorrelation function is curve fitted with the appropriate biological constrained model (reviewed in ²⁴) and a diffusion coefficient can be obtained. Increase in amplitude of $G(\tau)$ at larger correlation times is interpreted as binding events (Fig 2(c)).

FCS can provide quantitative data for modelling efforts. From the autocorrelation function, the average number of molecules within the volume can be calculated. Given that the dimension of the volume illuminated is known, the number of molecules can be converted into concentration ²⁴. Kinetic information of binding reactions to non fluorescent entities and aggregation processes can also be obtained, as these events will affect diffusion properties and thus will change the correlation time ²⁴.

Measuring Activity States in living cells

FRET- Fluorescence resonance energy transfer

FRET, using genetically- encoded fluorophores, has become a more commonly used approach to study signalling in the intact living cell²⁵. FRET is defined as the measurement of energy transfer between two fluorophores, a donor and an acceptor, with overlapping spectra but distinct emission peaks (Fig 3). The donor fluorophore, after being excited in the shorter wavelength range, will excite the acceptor of a longer wavelength range via energy transfer. Close proximity (in the angstrom range) of the two fluorophores, is essential for FRET (Fig 3(a)).

FRET signals can be detected in a several ways: sensitized acceptor emission (increase in acceptor fluorescence); decrease in donor fluorescence and decrease in donor fluorescence lifetime. In sensitized emission, the fluorescence of both the donor and the acceptor are monitored over time upon excitation of the donor. With FRET conditions, the ratio of the fluorescence of the acceptor to the donor increases. The sensitized emission FRET, albeit easy to measure, requires several corrections that must be applied post-imaging.

The most commonly used fluorescent proteins FRET pairs are cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) or their mutation variants mCerulean and Venus^{26, 27}. Several point mutations in these variants have improved their brightness, photostability and maturation rates. Yet, compared to fluorescent dyes, these genetically encoded fluorescent protein pairs are subpar FRET donors and acceptors. Given their broad excitation and emission curves, controls must be included to account for possible bleed through (Fig 3(b)). This bleed through can be due to a) the donor excitation wavelength weakly exciting the acceptor even in non-FRET conditions and b) overlapping donor and acceptor emission curves. Also, a certain amount of photobleaching during visualization of the two fluorophores is inevitable. Thus appropriate controls and corrections should be used when performing sensitized emission measurements²⁸.

A more dependable FRET technique is to track the decrease in donor fluorescence lifetime, since none of the bleed through issues occur^{29, 30}. In fluorescence lifetime imaging microscopy (FLIM), instead of monitoring the ratio of acceptor to donor fluorescence intensity, the time a single donor fluorophore takes to emit and return to its non-excited (or ground) state is measured. This time (or lifetime) is compared in the non-FRET-ing condition to the FRET-ing condition, where some of the energy is diverted to excite the acceptor and thus shortens the time the donor fluorophore spends in the excited state - reaching ground state faster. Thus an increase in FRET is measured as a decrease in fluorescence lifetime for the donor.

Two types of FRET probes have been developed: reporters of activity states and reporters of protein:protein interactions^{31, 32}. Protein protein interaction reporters, or intermolecular FRET reporters, consist of the two proteins of interest each tagged with a fluorophore (Fig 3(c)). When dimerization of the proteins of interest occurs, the two fluorophores come in close proximity and FRET signal increases. One of the caveats of this type of FRET reporters is that if the reporter components are not expressed in excess, they will interact with non-tagged partners, resulting in an increase in noise with no contribution to the FRET signal. Care must be taken when expressing intermolecular FRET reporters, as differences in the molar ratios of the two tagged units may skew the FRET signal. Some of these issues are mitigated in single polypeptide reporters. Reporters of activity state may contain a single protein domain, flanked by CFP and YFP, that will bind to the molecule of interest and this binding event will induce a change in the domain conformation. This change in conformation will bring in close proximity the fluorophores, leading to FRET. These single polypeptide, or intramolecular FRET reporters interact with a non-tagged activated protein, or a small molecule such as cAMP or Ca^{++} (Fig 3(c)). Other intramolecular FRET activity reporters may employ conserved amino acid sequences that will act as a pseudo substrate for an enzyme, such as a kinase or an acetylase^{33, 34}. Also in tandem will be a domain that will specifically bind to the modified pseudo-substrate. For example, a src activity FRET reporter involves a substrate peptide derived from the endogenous src substrate p130cas, along with a SH2 domain, a phospho-tyrosine binding motif³⁵. This binding event will decrease the distance between the two fluorophores and thus result in an increase in FRET. These types of reporters monitor the balance between both the activity of the modifying enzyme and the reverse reaction (catalysed by phosphatases or deacetylases, for example). Uncoupling the contribution of the two reactions may be done with the use of specific inhibitors for one of the enzymes during the FRET experiment. Activity reporters can also be targeted to specific subcellular regions by including targeting sequences or molecular zip codes³⁶. The tethered reporter can then be used to monitor compartment specific changes in signalling. Wang and co-workers have targeted src FRET biosensor to lipid rafts by adding a prenylation sequence derived from Lyn kinase, a known resident of lipid rafts³⁵. This allowed the temporal and spatial monitoring of src activation in response to force³⁵.

The number of FRET reporters available is rapidly increasing, making the probing of multiple signalling molecules within a network feasible (Fig 4). Reporters are able to measure levels of small diffusible molecules such as cAMP, Ca^{++} , cGMP, Cl^- , K^+ and NO or modified lipids such as PIP_2 , DAG and PIP_3 ³⁷⁻⁴³. Reporters are also available to measure changes in activity states for small GTPase such as Ras, Rap, Rac, Cdc42 and Rho or

heterotrimeric G-proteins such as Gs and Gq⁴⁴⁻⁴⁹. Enzyme activity is measured with pseudo-substrate reporters for kinases such as PKA, MAPK, AKT, Src, JNK, PKC and PKD^{35, 50-58}. Other pseudo-substrate reporters can measure proteolytic activity of caspases or proteases⁵⁹. Transcription factors reporters, as for NFkB and CREB are also available. Chromatin states can also be monitored by tracking modifications of histones^{33, 60}. Zhang and co-workers have developed a biosensor that can simultaneously monitor cAMP and PKA dynamics⁶¹.

Care must be taken to control for the possibility that the expression of any of these types of reporters may significantly compete with or hinder endogenous signalling. Titrating the expression of the reporter can control for this. Thus, all these techniques are only informative if the proper controls are included. The evaluation of published studies to obtain useful biological parameters for modelling should be done paying attention to the technical merits of the studies. Understanding the experimental techniques, and their limitations, allows the proper interpretation of the biological data and its translation into its mathematical formulation. For example, many of these reporters will have a narrow dynamic range of activity, since they are dependent on binding affinities.

Imaging driven insight—One of the most orchestrated events in the lifespan of a cell is its replication and division. During mitosis, all the genetic material must be doubled, segregated into opposite poles of the parental cell and divided into two identical daughter cells. Throughout this process the nuclear envelope disappears and the structural spatial cues that distinguish the nuclear from the cytosolic compartment are lost. It is during this time that spatial gradients of signaling molecules, such as Ran-GTP and Aurora kinase, maintain the spatial cues that play a key role in the coordination of the events underlying the cell cycle⁶².

Ran is a small G-protein regulated by the opposing actions of RCC1, a chromatin binding-GEF (guanine exchange factor) that catalyzes the GTP loading of Ran, and RanGAP, a cytoplasmic GAP (GTPase activating protein) that binds to Ran-GTP and stimulates its intrinsic GTPase activity to hydrolyze GTP to GDP. Due to the localization of RCC1 and RanGAP, Ran-GTP is concentrated around chromatin, while Ran-GDP is found mostly in the cell periphery. This Ran-GTP gradient was visualized by sensitized emission and by FLIM, using a FRET reporter⁶³⁻⁶⁵. These imaging studies have pinpointed to the multitude of roles Ran has throughout the cell cycle. During interphase, Ran-GTP gradient is essential for the bidirectional nucleo-cytoplasmic transport of cargo. Ran-GTP gradient is also vital for the assembly of the mitotic spindle at its proper location. During this process the cell lacks a nuclear delimited region, and Ran in its GDP and GTP form, are the positional cues maintaining the invisible cyto-nuclear boundary.

cAMP, the canonical small highly diffusible second messenger, is found in concentrated intracellular regions called microdomains. This is an example of the valuable insight provided by imaging experiments. Discovered in the 1960s, the cAMP pathway is probably one of the most thoroughly studied signalling pathways. cAMP is synthesized by membrane bound adenylyl cyclase and degraded by cytoplasmic phosphodiesterases. Traditional biochemical approaches used to measure cAMP relied on the radiolabeling and

homogenization of cells or tissue, allowing for quantitative monitoring of only the temporal aspect of cAMP signaling. Tsien and coworkers pioneered the monitoring of the spatial aspect of cAMP, using a FRET- based reporter to show quantitative measurements of cAMP in situ⁶⁶. Using a genetically encoded version of the reporter, Zacco and Pozzan discovered cAMP microdomains in myocytes⁶⁷. These studies have been expanded, by us and others, to show that cAMP is highly compartmentalized and this compartmentalization might be essential for signal specificity⁶⁸⁻⁷⁰.

Both examples described above highlight the rich organization of the cellular signalling. Imaging studies can be quantitative and show the non homogenous nature of signaling in real time. Unfortunately, just observing these gradients is not sufficient to answer how these non uniform distributions form and are maintained. Thus, the use of theoretical approaches is a great compliment to imaging studies as they allow the mechanistic dissection of these gradients.

Computational Approaches to Spatial Intracellular Biology

Three steps must be followed during the development of any type of dynamical model: assembly, calibration and validation^{71, 72}. Model assembly requires the translation of biochemical reactions into mathematical formulations^{73, 74}. The granularity (or amount of detail) and scope of the model will be dictated by the ultimate goal of the study. In some instances, a model that recapitulates biological phenomena using minimal number of reactions may be preferred over one containing all the known intricate details of the cellular event of interest. Including additional reactions will increase the number of parameters needed, and in some cases these parameters may not be available, making the model more difficult to constrain. The caveat of using a small number of reactions is a decrease in the model's predictive power, and overall mechanistic insight potential. For example, the small GTPase Ras is membrane associated by a palmitoyl moiety, and this involves a series of enzymatic reactions occurring at the endoplasmic reticulum and subsequent transport to the plasma membrane. In models with movement of Ras between organelles, including these reactions may be essential to recapitulate the dynamics of Ras^{75, 76}. In other cases, the simulation time period may be shorter than the half-life of Ras, and thus ignoring the processing of Ras may be valid^{77, 78}.

The next step, calibration, involves using experimental data to constrain the model⁷². Unknown parameters can be estimated using available software packages such as MATLAB, or Copasi⁷⁹. The type of experimental data used is input/output relationships, in the form of time courses and dose response curves after the addition of a ligand or other perturbations^{1, 2}. Parameter sensitivity analysis, where one or more parameters are varied and effect on model output is checked, is used to pinpoint those parameters that require the most accuracy. These parameters will have the greatest effect on the output of the model⁷¹. New computational techniques suggest that values of many model parameters may be determined all at once through a systematic comparison with experimental data⁸⁰. Finally, comparing simulation results to experimental data that was not used during the calibration step validates the model. The experimental data may involve perturbations, such as the use of siRNA to decrease the level of a protein, or a chemical inhibitor to reduce the activity of

an enzyme. These perturbations can be explicitly included in the model. For example an inhibitor may work by decreasing the enzyme's affinity for its substrate- thus in the model increasing the K_m of the reaction may be a good representation of the inhibition. The validation process can also be in the form of model predictions that drive biological testing. Novel relationships suggested by the model may be tested empirically. All calibrations are informative, even for experimental data that the model fails to replicate, since these highlight the need for revision of the model by changing parameters, assumptions or network topology (Fig 5a).

There are several types of dynamical models: Boolean logic models and Differential equation- based deterministic models and stochastic models. Boolean logic models require information about network topology, but do not need kinetic parameters, just logical operations (AND, OR) to describe the relationships between the components. Differential equation models describe biochemical reactions in a deterministic or stochastic framework. In the deterministic realm, reactions are calculated ignoring any influence of random events or noise. These models give consistently reproducible results given an initial set of parameters. If noise fluctuations cannot be ignored (as in the case of reactions occurring in very small volumes, or with very few molecules are involved), then the stochastic framework is required. Due to space constraints, stochastic modelling methods will not be covered, but are extensively reviewed elsewhere⁸¹⁻⁸³.

ODE-based models

The most common type of model is a system of coupled ordinary differential equations (ODEs) describing kinetic reactions with mass action representations. Michaelis-Menten approximations can also be used to describe enzymatic reactions. ODEs are the mathematical representation of biochemical reactions and calculate changes of concentrations as a function of time. The parameters needed are experimentally measurable numbers such as concentrations, reaction rates and binding affinities. Several available modelling platforms have solvers to calculate ODEs such as Jsim, MATLAB, COPASI or Virtual Cell.

The spatial aspect of signalling can be abstracted into ODE-based models by the inclusion of compartments. Compartments may represent physical membrane-delimited entities such as organelles or less defined regions, such as the sub-membrane space or peri-nuclear region of the cell⁸⁴. Each compartment can be defined in terms of volume, surface, and subset of molecules, permitting the representation of a heterogeneous environment (Fig 5b). Within a compartment, all molecules are uniformly distributed (well-mixed assumption). Diffusion coefficients and active transport are abstracted as transport rates that connect the compartments.

PDE-based models

The consideration of the space dimension, when modelling intracellular signalling, is a relatively recent occurrence. The compartmental ODE representation is not valid, and a system of coupled partial differential equations (PDEs) is required, if diffusion and cellular geometry need to be explicitly included. PDEs calculate the changes of concentration as a

function of time and space. Virtual Cell (Vcell.org) is a modelling platform that utilizes a uniform finite volume solver to calculate PDEs. Cellular geometries can be implemented into models, allowing the direct comparison with imaging data. VCell contains the flexibility of transforming an originally compartmental ODE model into a PDE model by assigning it a 2D or 3D geometry. Experimental microscope images can be converted into geometries. Images are segmented into grayscale values, with each gray value representing a compartment of defined structure, and each compartment identified by its unique gray value. Model compartments (originally defined just in terms of volume and surface area) can be assigned to cellular morphological sub-structures, such as the nucleus (Fig 5b). The whole geometry is then subdivided into a uniform mesh of sub-volumes, with the mesh resolution (number of sub-volumes per unit area) defined by the user. Each sub-volume should represent a region sufficiently small that any concentration differences within should be negligible. In the case of 3D geometries, a stack of segmented 2D images is used.

In addition to the parameters needed for an ODE-based model, diffusion coefficients must be assigned for each molecule included in the PDE model. FRAP and FCS studies can be used to obtain diffusion coefficients. Unfortunately, this type of parameter may not be readily available for all molecules, and must be sometimes approximated using known diffusion coefficients of other proteins along with their molecular weight as references. Comparison of reaction dynamics data obtained by FRET to simulations, may require the explicit description in the model of the reaction(s) involving the exogenously expressed reporter.

Insights from spatial computational models—Computational models have highlighted several key mechanistic insights that underlie the formation of inhomogeneous signalling. Seminal modeling work by Kholodenko and colleagues, explored how spatial segregation of antagonistic biochemical reactions (such as a membrane-bound kinase and cytosolic phosphatase acting on a diffusible substrate) is sufficient for the formation of intracellular gradients⁸⁵⁻⁸⁷. This segregation of activities seems to be the basis for cAMP microdomains and Ran-GTP gradients. Given how most inputs into the cell have to be processed by membrane bound receptors, and get deactivated in the cytosolic milieu, cellular shape and size are also contributors to the formation of observed signaling microdomains for MAPK and cdc42^{88, 89}. In these examples, direct comparison of FRET based measurements and simulation predictions have highlighted important factors that contribute to gradients formation but are not experimentally feasible to modulate. Other PDEs-based models have confirmed possible mechanisms that give rise to the observed inhomogeneities. Internalized G-protein coupled receptors, thought to be desensitized non signalling entities, do continue signalling and the internalized-derived signals lead to distinct cellular outcomes than those originating at the plasma membrane⁹⁰.

Conclusion

The goal of this review was to summarize some of the experimental and theoretical approaches currently available to study the inhomogeneous nature of cell signalling. The review described the imaging techniques used to obtain diffusion coefficients, concentrations and reaction dynamics from live cells. The steps needed to construct ODEs

and PDEs based kinetic models and compare them to data were also discussed. Computational models of spatial cellular biology can be used as hypothesis producing tools and direct experimental efforts. This approach, of combining imaging and mathematical framework, has provided valuable insight into mechanistic details that would not be possible otherwise. Models have also been used to further confirm possible mechanisms suggested by experimental approaches. Both cases highlight the complementary nature of imaging and computational modeling.

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References

1. Neves SR, Iyengar R. Modeling of signaling networks. *Bioessays*. 2002; 24(12):1110–1117. [PubMed: 12447976]
2. Neves SR, Iyengar R. Models of spatially restricted biochemical reaction systems. *J Biol Chem*. 2009; 284(9):5445–5449. [PubMed: 18940805]
3. Mor A, Campi G, Du G, Zheng Y, Foster DA, et al. The lymphocyte function-associated antigen-1 receptor costimulates plasma membrane Ras via phospholipase D2. *Nat Cell Biol*. 2007; 9(6):713–719. [PubMed: 17486117]
4. Fuller BG, Lampson MA, Foley EA, Rosasco-Nitcher S, Le KV, et al. Midzone activation of aurora B in anaphase produces an intracellular phosphorylation gradient. *Nature*. 2008; 453(7198):1132–1136. [PubMed: 18463638]
5. Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *J Cell Comp Physiol*. 1962; 59:223–239. [PubMed: 13911999]
6. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene*. 1992; 111(2):229–233. [PubMed: 1347277]
7. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. *Science (New York, N Y)*. 1994; 263(5148):802–805.
8. Li X, Zhao X, Fang Y, Jiang X, Duong T, et al. Generation of destabilized green fluorescent protein as a transcription reporter. *J Biol Chem*. 1998; 273(52):34970–34975. [PubMed: 9857028]
9. Yu J, Xiao J, Ren X, Lao K, Xie XS. Probing gene expression in live cells, one protein molecule at a time. *Science*. 2006; 311(5767):1600–1603. [PubMed: 16543458]
10. Terskikh A, Fradkov A, Ermakova G, Zaraisky A, Tan P, et al. “Fluorescent timer”: protein that changes color with time. *Science*. 2000; 290(5496):1585–1588. [PubMed: 11090358]
11. Tsien RY. Constructing and exploiting the fluorescent protein paintbox (Nobel Lecture). *Angew Chem Int Ed Engl*. 2009; 48(31):5612–5626. [PubMed: 19565590]
12. Kogure T, Karasawa S, Araki T, Saito K, Kinjo M, Miyawaki A. A fluorescent variant of a protein from the stony coral *Montipora* facilitates dual-color single-laser fluorescence cross-correlation spectroscopy. *Nat Biotechnol*. 2006; 24(5):577–581. [PubMed: 16648840]
13. Kneen M, Farinas J, Li Y, Verkman AS. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophys J*. 1998; 74(3):1591–1599. [PubMed: 9512054]
14. Miesenböck G, De Angelis DA, Rothman JE. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 1998; 394(6689):192–195. [PubMed: 9671304]
15. Jayaraman S, Haggie P, Wachter RM, Remington SJ, Verkman AS. Mechanism and cellular applications of a green fluorescent protein-based halide sensor. *J Biol Chem*. 2000; 275(9):6047–6050. [PubMed: 10692389]
16. Dooley CT, Dore TM, Hanson GT, Jackson WC, Remington SJ, Tsien RY. Imaging dynamic redox changes in mammalian cells with green fluorescent protein indicators. *J Biol Chem*. 2004; 279(21):22284–22293. [PubMed: 14985369]

17. Patterson GH, Lippincott-Schwartz J. Selective photolabeling of proteins using photoactivatable GFP. *Methods*. 2004; 32(4):445–450. [PubMed: 15003607]
18. Lippincott-Schwartz J, Snapp E, Kenworthy A. Studying protein dynamics in living cells. *Nat Rev Mol Cell Biol*. 2001; 2(6):444–456. [PubMed: 11389468]
19. Reits EA, Neefjes JJ. From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat Cell Biol*. 2001; 3(6):E145–147. [PubMed: 11389456]
20. McNally JG. Quantitative FRAP in analysis of molecular binding dynamics in vivo. *Methods Cell Biol*. 2008; 85:329–351. [PubMed: 18155469]
21. Patterson GH, Lippincott-Schwartz J. A photoactivatable GFP for selective photolabeling of proteins and cells. *Science*. 2002; 297(5588):1873–1877. [PubMed: 12228718]
22. Groves JT, Parthasarathy R, Forstner MB. Fluorescence imaging of membrane dynamics. *Annu Rev Biomed Eng*. 2008; 10:311–338. [PubMed: 18429702]
23. Medina MA, Schwille P. Fluorescence correlation spectroscopy for the detection and study of single molecules in biology. *Bioessays*. 2002; 24(8):758–764. [PubMed: 12210537]
24. Bacia K, Kim SA, Schwille P. Fluorescence cross-correlation spectroscopy in living cells. *Nat Methods*. 2006; 3(2):83–89. [PubMed: 16432516]
25. Piston DW, Kremers GJ. Fluorescent protein FRET: the good, the bad and the ugly. *Trends Biochem Sci*. 2007; 32(9):407–414. [PubMed: 17764955]
26. Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol*. 2004; 22(4):445–449. [PubMed: 14990965]
27. Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol*. 2002; 20(1):87–90. [PubMed: 11753368]
28. van Rheenen J, Langeslag M, Jalink K. Correcting confocal acquisition to optimize imaging of fluorescence resonance energy transfer by sensitized emission. *Biophys J*. 2004; 86(4):2517–2529. [PubMed: 15041688]
29. Bastiaens PI, Squire A. Fluorescence lifetime imaging microscopy: spatial resolution of biochemical processes in the cell. *Trends Cell Biol*. 1999; 9(2):48–52. [PubMed: 10087617]
30. Harpur AG, Wouters FS, Bastiaens PI. Imaging FRET between spectrally similar GFP molecules in single cells. *Nat Biotechnol*. 2001; 19(2):167–169. [PubMed: 11175733]
31. Miyawaki A. Visualization of the spatial and temporal dynamics of intracellular signaling. *Dev Cell*. 2003; 4(3):295–305. [PubMed: 12636912]
32. Miyawaki A, Tsien RY. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol*. 2000; 327:472–500. [PubMed: 11045004]
33. Sasaki K, Ito T, Nishino N, Khochbin S, Yoshida M. Real-time imaging of histone H4 hyperacetylation in living cells. *Proc Natl Acad Sci U S A*. 2009; 106(38):16257–16262. [PubMed: 19805290]
34. Aye-Han NN, Ni Q, Zhang J. Fluorescent biosensors for real-time tracking of post-translational modification dynamics. *Curr Opin Chem Biol*. 2009; 13(4):392–397. [PubMed: 19682946]
35. Wang Y, Botvinick EL, Zhao Y, Berns MW, Usami S, et al. Visualizing the mechanical activation of Src. *Nature*. 2005; 434(7036):1040–1045. [PubMed: 15846350]
36. VanEngelenburg SB, Palmer AE. Fluorescent biosensors of protein function. *Curr Opin Chem Biol*. 2008; 12(1):60–65. [PubMed: 18282482]
37. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, et al. Fluorescent indicators for Ca²⁺ based on green fluorescent proteins and calmodulin. *Nature*. 1997; 388(6645):882–887. [PubMed: 9278050]
38. Ananthanarayanan B, Ni Q, Zhang J. Signal propagation from membrane messengers to nuclear effectors revealed by reporters of phosphoinositide dynamics and Akt activity. *Proc Natl Acad Sci U S A*. 2005; 102(42):15081–15086. [PubMed: 16214892]
39. DiPilato LM, Cheng X, Zhang J. Fluorescent indicators of cAMP and Epac activation reveal differential dynamics of cAMP signaling within discrete subcellular compartments. *Proc Natl Acad Sci U S A*. 2004; 101(47):16513–16518. [PubMed: 15545605]

40. Honda A, Adams SR, Sawyer CL, Lev-Ram V, Tsien RY, Dostmann WR. Spatiotemporal dynamics of guanosine 3',5'-cyclic monophosphate revealed by a genetically encoded, fluorescent indicator. *Proc Natl Acad Sci U S A*. 2001; 98(5):2437–2442. [PubMed: 11226257]
41. Mank M, Santos AF, Drenth S, Mrcic-Flogel TD, Hofer SB, et al. A genetically encoded calcium indicator for chronic in vivo two-photon imaging. *Nat Methods*. 2008; 5(9):805–811. [PubMed: 19160515]
42. Sato M, Ueda Y, Takagi T, Umezawa Y. Production of PtdInsP3 at endomembranes is triggered by receptor endocytosis. *Nat Cell Biol*. 2003; 5(11):1016–1022. [PubMed: 14528311]
43. Nikolaev VO, Bunemann M, Hein L, Hannawacker A, Lohse MJ. Novel single chain cAMP sensors for receptor-induced signal propagation. *J Biol Chem*. 2004; 279(36):37215–37218. [PubMed: 15231839]
44. Seth A, Otomo T, Yin HL, Rosen MK. Rational design of genetically encoded fluorescence resonance energy transfer-based sensors of cellular Cdc42 signaling. *Biochemistry*. 2003; 42(14):3997–4008. [PubMed: 12680752]
45. Mochizuki N, Yamashita S, Kurokawa K, Ohba Y, Nagai T, et al. Spatio-temporal images of growth-factor-induced activation of Ras and Rap1. *Nature*. 2001; 411(6841):1065–1068. [PubMed: 11429608]
46. Bunemann M, Frank M, Lohse MJ. Gi protein activation in intact cells involves subunit rearrangement rather than dissociation. *Proc Natl Acad Sci U S A*. 2003; 100(26):16077–16082. [PubMed: 14673086]
47. Hein P, Frank M, Hoffmann C, Lohse MJ, Bunemann M. Dynamics of receptor/G protein coupling in living cells. *EMBO J*. 2005; 24(23):4106–4114. [PubMed: 16292347]
48. Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, et al. Gs activation is time-limiting in initiating receptor-mediated signaling. *J Biol Chem*. 2006; 281(44):33345–33351. [PubMed: 16963443]
49. Itoh RE, Kurokawa K, Ohba Y, Yoshizaki H, Mochizuki N, Matsuda M. Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol*. 2002; 22(18):6582–6591. [PubMed: 12192056]
50. Sasaki K, Sato M, Umezawa Y. Fluorescent indicators for Akt/protein kinase B and dynamics of Akt activity visualized in living cells. *J Biol Chem*. 2003; 278(33):30945–30951. [PubMed: 12773546]
51. Allen MD, Zhang J. Subcellular dynamics of protein kinase A activity visualized by FRET-based reporters. *Biochem Biophys Res Commun*. 2006; 348(2):716–721. [PubMed: 16895723]
52. Fosbrink M, Aye-Han NN, Cheong R, Levchenko A, Zhang J. Visualization of JNK activity dynamics with a genetically encoded fluorescent biosensor. *Proc Natl Acad Sci U S A*. 2010; 107(12):5459–5464. [PubMed: 20212108]
53. Newman RH, Zhang J. Visualization of phosphatase activity in living cells with a FRET-based calcineurin activity sensor. *Mol Biosyst*. 2008; 4(6):496–501. [PubMed: 18493642]
54. Gao X, Zhang J. Spatiotemporal analysis of differential Akt regulation in plasma membrane microdomains. *Mol Biol Cell*. 2008; 19(10):4366–4373. [PubMed: 18701703]
55. Kunkel MT, Ni Q, Tsien RY, Zhang J, Newton AC. Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. *J Biol Chem*. 2005; 280(7):5581–5587. [PubMed: 15583002]
56. Violin JD, Zhang J, Tsien RY, Newton AC. A genetically encoded fluorescent reporter reveals oscillatory phosphorylation by protein kinase C. *J Cell Biol*. 2003; 161(5):899–909. [PubMed: 12782683]
57. Kunkel MT, Toker A, Tsien RY, Newton AC. Calcium-dependent regulation of protein kinase D revealed by a genetically encoded kinase activity reporter. *J Biol Chem*. 2007; 282(9):6733–6742. [PubMed: 17189263]
58. Harvey CD, Ehrhardt AG, Cellurale C, Zhong H, Yasuda R, et al. A genetically encoded fluorescent sensor of ERK activity. *Proc Natl Acad Sci U S A*. 2008; 105(49):19264–19269. [PubMed: 19033456]

59. Hellwig CT, Kohler BF, Lehtivarjo AK, Dussmann H, Courtney MJ, et al. Real time analysis of tumor necrosis factor-related apoptosis-inducing ligand/cycloheximide-induced caspase activities during apoptosis initiation. *J Biol Chem*. 2008; 283(31):21676–21685. [PubMed: 18522940]
60. Kimura H, Hayashi-Takanaka Y, Yamagata K. Visualization of DNA methylation and histone modifications in living cells. *Curr Opin Cell Biol*. 2010; 22(3):412–418. [PubMed: 20207127]
61. Ni Q, Ganesan A, Aye-Han N-N, Gao X, Allen MD, et al. Signaling diversity of PKA achieved via a Ca^{2+} -cAMP-PKA oscillatory circuit. *Nat Chem Biol*. 2011; 7(1):34–40. [PubMed: 21102470]
62. Fuller BG. Self-organization of intracellular gradients during mitosis. *Cell Div*. 2010; 5(1):5. [PubMed: 20181052]
63. Kalab P, Heald R. The RanGTP gradient - a GPS for the mitotic spindle. *J Cell Sci*. 2008; 121(Pt 10):1577–1586. [PubMed: 18469014]
64. Kalab P, Pralle A, Isacoff EY, Heald R, Weis K. Analysis of a RanGTP-regulated gradient in mitotic somatic cells. *Nature*. 2006; 440(7084):697–701. [PubMed: 16572176]
65. Kalab P, Weis K, Heald R. Visualization of a Ran-GTP gradient in interphase and mitotic *Xenopus* egg extracts. *Science*. 2002; 295(5564):2452–2456. [PubMed: 11923538]
66. Bacskaï BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, et al. Spatially resolved dynamics of cAMP and protein kinase A subunits in *Aplysia* sensory neurons. *Science*. 1993; 260(5105):222–226. [PubMed: 7682336]
67. Zaccolo M, Pozzan T. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science*. 2002; 295(5560):1711–1715. [PubMed: 11872839]
68. Castro LRV, Gervasi N, Guiot E, Cavellini L, Nikolaev VO, et al. Type 4 phosphodiesterase plays different integrating roles in different cellular domains in pyramidal cortical neurons. *Journal of Neuroscience*. 2010; 30(17):6143–6151. [PubMed: 20427672]
69. Ni Q, Ganesan A, Aye-Han NN, Gao X, Allen MD, et al. Signaling diversity of PKA achieved via a Ca^{2+} -cAMP-PKA oscillatory circuit. *Nature Chemical Biology*. 2011; 7(1):34–40.
70. Neves SR, Tsokas P, Sarkar A, Grace EA, Rangamani P, et al. Cell Shape and Negative Links in Regulatory Motifs Together Control Spatial Information Flow in Signaling Networks. *Cell*. 2008; 133(4):666–680. [PubMed: 18485874]
71. Aldridge BB, Burke JM, Lauffenburger DA, Sorger PK. Physicochemical modelling of cell signalling pathways. *Nat Cell Biol*. 2006; 8(11):1195–1203. [PubMed: 17060902]
72. Chen WW, Niepel M, Sorger PK. Classic and contemporary approaches to modeling biochemical reactions. *Genes Dev*. 2010; 24(17):1861–1875. [PubMed: 20810646]
73. Weng G, Bhalla US, Iyengar R. Complexity in biological signaling systems. *Science*. 1999; 284(5411):92–96. [PubMed: 10102825]
74. Bhalla US, Iyengar R. Emergent properties of networks of biological signaling pathways. *Science*. 1999; 283(5400):381–387. [PubMed: 9888852]
75. Eungdamrong NJ, Iyengar R. Compartment-specific feedback loop and regulated trafficking can result in sustained activation of Ras at the Golgi. *Biophys J*. 2007; 92(3):808–815. [PubMed: 17098795]
76. Lorentzen A, Kinkhabwala A, Rocks O, Vartak N, Bastiaens PI. Regulation of Ras localization by acylation enables a mode of intracellular signal propagation. *Sci Signal*. 2010; 3(140):ra68. [PubMed: 20858867]
77. Schoeberl B, Eichler-Jonsson C, Gilles ED, Muller G. Computational modeling of the dynamics of the MAP kinase cascade activated by surface and internalized EGF receptors. *Nat Biotechnol*. 2002; 20(4):370–375. [PubMed: 11923843]
78. Kholodenko BN, Demin OV, Moehren G, Hoek JB. Quantification of short term signaling by the epidermal growth factor receptor. *J Biol Chem*. 1999; 274(42):30169–30181. [PubMed: 10514507]
79. Mendes P, Hoops S, Sahle S, Gauges R, Dada J, Kummer U. Computational modeling of biochemical networks using COPASI. *Methods Mol Biol*. 2009; 500:17–59. [PubMed: 19399433]
80. Sarkar AX, Sobie EA. Regression analysis for constraining free parameters in electrophysiological models of cardiac cells. *PLoS Comput Biol*. 2010; 6(9):e1000914. [PubMed: 20824123]

81. Takahashi K, Arjunan SN, Tomita M. Space in systems biology of signaling pathways--towards intracellular molecular crowding in silico. *FEBS Lett.* 2005; 579(8):1783–1788. [PubMed: 15763552]
82. Wilkinson DJ. Stochastic modelling for quantitative description of heterogeneous biological systems. *Nat Rev Genet.* 2009; 10(2):122–133. [PubMed: 19139763]
83. Cheong R, Paliwal S, Levchenko A. Models at the single cell level. *Wiley Interdiscip Rev Syst Biol Med.* 2010; 2(1):34–48. [PubMed: 20836009]
84. Oliveira RF, Terrin A, Di Benedetto G, Cannon RC, Koh W, et al. The role of type 4 phosphodiesterases in generating microdomains of cAMP: large scale stochastic simulations. *PLoS One.* 2010; 5(7):e11725. [PubMed: 20661441]
85. Kholodenko BN. MAP kinase cascade signaling and endocytic trafficking: a marriage of convenience? *Trends Cell Biol.* 2002; 12(4):173–177. [PubMed: 11978536]
86. Kholodenko BN, Brown GC, Hoek JB. Diffusion control of protein phosphorylation in signal transduction pathways. *Biochem J.* 2000; 350(Pt 3):901–907. [PubMed: 10970807]
87. Brown GC, Kholodenko BN. Spatial gradients of cellular phospho-proteins. *FEBS Lett.* 1999; 457(3):452–454. [PubMed: 10471827]
88. Meyers J, Craig J, Odde DJ. Potential for control of signaling pathways via cell size and shape. *Curr Biol.* 2006; 16(17):1685–1693. [PubMed: 16950104]
89. Neves SR, Tsokas P, Sarkar A, Grace EA, Rangamani P, et al. Cell shape and negative links in regulatory motifs together control spatial information flow in signaling networks. *Cell.* 2008; 133(4):666–680. [PubMed: 18485874]
90. Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, et al. Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. *PLoS Biol.* 2009; 7(8):e1000172. [PubMed: 19688034]

Figure 1.

Fluorescence Recovery After Photobleaching. (a) Schematic of a typical FRAP experiment. A cell expressing a fluorescent-tagged protein is photobleached in a defined area (1, red square). The defined area is photobleached until no fluorescence is observed (2). The recovery of fluorescence is monitored (3, 4). (b) The corresponding fluorescence trace is analyzed to determine the mobile and immobile fractions, and the equilibration half-time.

Figure 2.

Fluorescence Correlation Spectroscopy. (a) The movement of a fluorescent-tagged proteins in and out of a small illuminated volume is monitored. (b) (i) This results in multiple fluctuations in the fluorescence intensity over time. (ii) Autocorrelation analysis of fluctuations over time; $G(\tau)$ is the amplitude of the correlation and τ is the correlation time. (c) Changes in the autocorrelation function represent changes in (i) concentration or (ii) diffusion.

Figure 3.

Fluorescence Resonance Energy Transfer. FRET occurs when two fluorophores are in close proximity (a) and have overlapping spectra (b). Blue represents the donor, yellow represents the acceptor. Excitation is depicted as the dashed line, while emission is the solid line. In (b) the gray block presents the possible cross excitation of both the donor and the acceptor. The golden box represents the residual emission of the donor that must be subtracted from the FRET signal. (c) Schematic of the types of FRET probes available. (i) intermolecular reporters; (ii) intramolecular reporters (iii) reaction reporters

Figure 4.

Imaging reporters available for Signaling Proteins and Second messengers. Arrows depict signal flow. All of the molecules depicted here have available imaging reporters, allowing the monitoring of their levels of activity states. Small GTPases are shown as blue circles, while heterotrimeric G-proteins are green. Enzymes, such as kinases, are shown as orange circles. Transcription factors are red. Histones are shown as teal circles.

Figure 5.

(a) Flow diagram of the steps needed to develop a computational model. Model assembly requires the inclusion of the biochemical reaction scheme, the known parameters associated with the reactions, and the assumptions of the model. These steps will rely on previous knowledge available. Model calibration entails the use of experimental input:output relations to constrain any unknown parameters. Model validation involves the use of experimental data to probe the correctness of the model. The experimental data may have been a result of a model prediction. The iterative nature of modelling is shown, if the model fails to replicate the biological phenomena, then the steps of model assembly and calibration have to be revised. (b) ODE and PDE-based models. Biochemical reactions are represented in a mathematical formulation and placed in their proper compartments. Compartments are mapped to geometries derived from microscopic images. Model simulations lead to predictions that can be compared to experimental data.