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## **Endocytosis and signalling: a meeting with mathematics**

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## **Summary**

Although endocytosis has traditionally been understood as a signal attenuation mechanism, an emerging view considers endocytosis as an integral part of signal propagation and processing. On the short time scale, trafficking of endocytic vesicles contributes to signal propagation from the surface to distant targets, with bi-directional communication between signalling and trafficking. Mathematical modelling helps combine the mechanistic, molecular knowledge with rigorous analysis of the complex output dynamics of endocytosis in time and space. Simulations reveal novel roles for endocytosis, including the control of cell polarity, enhancing the spatial signal propagation, and controlling the signal magnitudes, kinetics, and synchronization with stimulus dynamics.

## Introduction

Endocytosis is a main process by which cells transport extracellular and plasma membrane bound entities into the cell interior. Although there are many intricate endocytic mechanisms (Bareford and Swaan, 2007; Benmerah and Lamaze, 2007; Jones, 2007; Rizzoli and Jahn, 2007), the formation of endocytic vesicles can be illustrated in terms of two simple component processes (Fig. 1). First, a part of the plasma membrane invaginates, and, second, it pinches off. The internalized endocytic vesicle, now within the cell and separate from the cell membrane, has in its lumen what was previously extracellular material, and has on its cytosolic surface what was previously cell membrane-bound and cytosolic. After internalization, cellular trafficking machinery transports the vesicle and its cargo to the appropriate cellular locations through a network of specialized organelles, including early endosomes, recycling endosomes, late endosomes, multi-vesicular bodies, and lysosomes (Hicke and Dunn, 2003; Sorkin and Goh, 2009; Williams and Urbe, 2007).

While the central importance of endocytosis in cell biology cannot be understated, of particular interest in this review is the role of endocytosis in signal transduction. Traditionally, endocytosis was regarded as a simple signal attenuation mechanism, as it removes signals from the extracellular space and receptors from the cell surface. These processes not only terminate signalling, but also desensitize the cell and prepare it for subsequent signals. Numerous recent studies, however, have brought about a more complex view: endocytosis and trafficking play a central role in signal propagation and specificity by regulating both the dynamics and localization of signalling (Di Fiore and De Camilli, 2001; Kholodenko, 2002; Polo and Di Fiore, 2006; Sorkin and Von Zastrow, 2002; von Zastrow and Sorkin, 2007). For example, the small G-protein Ras, whose constitutive activation drives a number of human malignancies, can be activated by receptor tyrosine kinases not only at the cell membrane, but also on the membranes of various endosomal structures (Haugh *et al*, 1999a; Jiang and Sorkin, 2002; Li *et al*, 2005). In some cases, such as vascular endothelial growth factor (VEGF)-induced extracellular-regulated kinase (ERK) activation, full signal propagation depends upon endocytosis (Lampugnani *et al*, 2006). In fact, for some signals that emanate at the cell surface, endocytosis may be the only way to reach distant cellular locations, such as the nucleus. Indeed, signal deactivation during diffusion in the cytoplasm can cause precipitous signalling gradients and very low signal magnitudes near the target (Brown and Kholodenko, 1999; Kholodenko, 2003). Such gradients of protein active forms have been observed for the small GTPase Ran (Kalab *et al*, 2002), phosphorylated stathmin oncoprotein 18 (Niethammer *et al*, 2004), and the yeast MAPK Fus3 (Maeder *et al*, 2007). Endosomal trafficking is even more crucial for signal propagation over distances greater than ~10-100  $\mu\text{m}$ , when diffusion is unsatisfactorily slow. Such situations arise in signal propagation from the plasma membrane to the nucleus in large cells, e.g., *Xenopus* oocytes (~ 1 mm), or in transport of nerve growth factor (NGF) survival signals from distal axon terminals to the soma (1 cm – 1 m). For the long distance transport, molecular motor-driven trafficking of endosomes or protein complexes not only accelerates signal propagation relative to diffusion, but association of signals with specific proteins can also help protect signals from deactivation (Howe and Mobley, 2004; Perlson *et al*, 2005; Perlson *et al*, 2006). In terms of localization, some MAPK cascade scaffolds are preferentially localized to either plasma or endosomal membranes, which could lead to different signalling outcomes depending on where the signal originates (Hancock, 2003; Kolch, 2005). Furthermore, the access to membrane bound substrates that are critical for signalling, such as phosphoinositols, can be regulated via endocytosis and trafficking (Haugh, 2002; Haugh *et al*, 1999b).

Not only does endocytosis control signalling, but signalling also regulates endocytosis, acting in a bi-directional manner (Von Zastrow and Sorkin, 2007). The classical

epidermal growth factor (EGF) receptor signalling system, for example, regulates endocytosis in several ways. First, EGF binding to its receptor (EGFR) causes a rapid increase in the rate of EGFR internalization (Lund *et al*, 1990). This increase is mediated by EGFR phosphorylation, recruitment of adaptor proteins such as Grb2, and activation of Cbl that ubiquitylates EGFR and possibly other endocytosis-related proteins (Huang *et al*, 2006). These events are followed by the recruitment of clathrin-coated pit proteins, e.g., epsin and AP-2, thus causing the increased endocytosis rate (Sorkin and Goh, 2008). Second, EGFR can regulate localization of the Rab family GTPases, which are crucial for directing traffic along the endocytic pathway and defining the identity of specific endosomal compartments (Rink *et al*, 2005). As the molecular machinery that underlies endocytosis has become further elucidated, more bi-directional links have been uncovered, which has led to an emerging view that signalling and endocytic systems are not distinct, but rather are interacting, co-evolving systems (Polo *et al*, 2006; von Zastrow *et al*, 2007).

Over the past decade, signal transduction research has been infused and enhanced with systems biology methodologies, endocytosis-related signal transduction being no exception (Kholodenko, 2006; Shankaran *et al*, 2007a; Wiley *et al*, 2003). The distinct feature of systems biology approaches when compared to traditional research is the development and use of mathematical and computational models applied to the biological system of interest (Kholodenko *et al*, 1999). A main advantage of using a mathematical model is that it allows one to analyze the system in ways not experimentally possible, whereas predictive, *in silico* simulations of the system output behaviour are experimentally testable. Moreover, these predictions are consistent with all the data and mechanistic information incorporated in the model, and this insight likely would not have been conceived merely from the data. Another advantage of using a mathematical model is that it can handle and interpret the bewildering complexity of signal transduction.

Although mathematical and computational modelling can be conceptually new to biologists and may raise doubts, mathematical models generally serve the same purposes as biological models, such as knockout mice or cell lines. For example, to understand a disease process occurring *in the human body*, most research is done *in cell lines and animal models*, rather than in the true system of interest. Although these “models” are not identical to the human body, they retain key features of the system of interest. To use such models successfully, one must first understand how the model is different from the real system (and therefore what research questions can and cannot be addressed) and then whether the new knowledge gained by using the model applies to the real system. When put in this context, a mathematical model is just another layer of abstraction from the system of interest; it allows one to address questions that are impossible or very difficult to answer using traditional biological models.

This review focuses on how mathematical modelling and systems biology help us understand endocytosis itself and its role in signal transduction. In the context of signal transduction, we will address here receptor-mediated endocytosis, a process where the binding of extracellular ligands to transmembrane receptors facilitates their internalization. Receptor-mediated endocytosis and its modelling have been focused on five classical model systems: EGF, Vitellogenin (Vtg), transferrin (Tf), low density lipoprotein (LDL), and insulin/glucose transporter (Glut). From a biological perspective, the EGF system is most relevant to cancer-related signal transduction. From a mathematical perspective, however, all five classical endocytic systems can be described by similar equations. Consequently, insight drawn from, for example, a Tf model may be applicable to the EGF system. Different models for multiple receptor-mediated endocytosis systems that have been developed over the past three decades are presented and grouped here according to the mathematical features of the model, rather than the biological system. Consequently, the mathematical commonalities

between the systems become apparent. As many mathematical modelling concepts can be unfamiliar to the general reader, we also include short sections on mathematical modelling fundamentals. Overall, although modelling has contributed to the understanding of endocytosis, much work remains to incorporate the ever increasing knowledge on molecular mechanisms into models.

## Mathematical Modelling Fundamentals

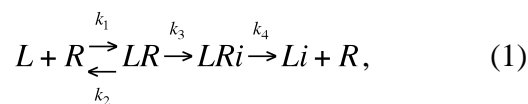
The overall modelling process can be subdivided into three repeated tasks:

1. Model Development: Converting Biological Knowledge into Equations
2. Parameter Estimation: Determining Values of Unknown Model Parameters
3. Model Validation: Comparing Model Predictions to Independent Experimental Data

The process is iterative, in that Step 3 inevitably leads to new biological knowledge that the model cannot describe, bringing the modeller back to Step 1. Below we describe these tasks and illustrate them with a simple endocytosis modelling example.

*Model Development: Converting Biological Knowledge into Equations.* The majority of endocytosis and signal transduction models are based on systems of ordinary differential equations (ODEs) and/or partial differential equations (PDEs). ODE models naturally arise from the description of processes where quantities of interest are changing with respect to time in a quantifiable manner. PDE models arise from extending the temporal description of dynamic processes into space. Here, we illustrate the process of converting biological knowledge into a system of ODEs with a simple example, while the basics of PDE models are described in a subsequent section. This example illustrates that when ligand is in excess of receptors, such as the case for LDL, ligand-induced endocytosis and recycling can be analogous to traditional enzyme kinetics (Harwood and Pellarin, 1997).

Biological processes involved in ligand-induced endocytosis can schematically be presented as the following simplified kinetic diagram (chemical reaction Scheme 1). In the first reaction, extracellular ligand  $L$  binds (with rate constant  $k_1$ ) and dissociates (with rate constant  $k_2$ ) from surface receptor  $R$ . The second reaction (Scheme 1) simplifies the complex process of internalization of the surface ligand-receptor complex  $LR$  as a single step with the rate constant  $k_3$ . The internalized ligand-receptor complex  $LRi$  breaks apart in the third reaction (rate constant  $k_4$ ), with the internalized ligand  $Li$  remaining inside the cell and the receptor recycling to the surface,



Although scheme 1 resembles the kinetic diagram of an enzyme reaction where the substrate ( $L$ ) is converted to the product ( $Li$ ) by the enzyme ( $R$ ), special conditions must be fulfilled in order to extend this similarity to the observed kinetic behaviours. The reaction rates of the processes,  $v_i$ , which describe how quickly the process is occurring, can be written according to the law of mass action as follows,

$$v_1 = k_1[L][R]; \quad v_2 = k_2[LR]; \quad v_3 = k_3[LR]; \quad v_4 = k_4[LRi], \quad (2)$$

Here square brackets denote species concentrations with respect to the total system volume.

The rate of change of a species concentration is the sum of all reaction rates that produce that species, minus all reaction rates that consume that species. For each species in Scheme 1, this verbal definition is stated mathematically in terms of ODEs as follows,

$$\begin{aligned}
\frac{d[R]}{dt} &= -v_1 + v_2 + v_4; & \frac{d[LR]}{dt} &= v_1 - v_2 - v_3; \\
\frac{d[LRi]}{dt} &= v_3 - v_4; & \frac{d[Li]}{dt} &= v_4;
\end{aligned} \tag{3}$$

where  $t$  denotes time. Eq. 3 is a system of ODEs that mathematically describes the four biological processes listed above. The dynamics for  $L$  are not considered, since as stated above, for this example we are only concerned with situations where ligand is in excess. If the dynamics of  $L$  were considered, however, the system of ODEs would be non-linear (as is standard in signal transduction), because then in the rate  $v_1$  two species concentrations would be multiplied by each other. All other rates are first-order, linear processes.

For many endocytic systems, the rate of internalized ligand accumulation,  $d[Li]/dt$ , or  $v_4$ , is of particular interest. Since this rate only depends explicitly on  $[LRi]$ , we now focus on deriving an expression for  $[LRi]$  in terms of experimentally accessible quantities.

Experimental observations suggest that in some situations, particularly when extracellular ligand is abundant, the rate-limiting step of the endocytic process is internalization (step 3) (Harwood *et al*, 1997). In such a scenario, the ligand binding/dissociation processes will quickly replace any ligand-receptor complexes lost from the plasma membrane via internalization. This creates a “pseudo-steady-state” situation for  $[LR]$ , where the number of ligand-receptor complexes on the cell surface is essentially constant over long time scales, where the long time scale is defined as the time scale of internalization. Mathematically, this is written as

$$\frac{d[LR]}{dt} \approx 0 \Rightarrow v_1 = v_2 + v_3 \tag{4}$$

Along similar lines, the rate of receptor recycling, as it is downstream from the rate-limiting step, can go only as fast as the endocytosis rate. Therefore, over long times scales, the rate of receptor recycling will be equal to the rate of endocytosis, giving a pseudo steady-state for  $[LRi]$  as well,

$$v_3 \approx v_4 \Rightarrow \frac{d[LRi]}{dt} \approx 0 \tag{5}$$

Based on Eqs. 4 and 5, we can derive an expression for  $[LRi]$  and therefore  $d[Li]/dt$ . First, by substituting the rate expressions from Eq. 2 into Eq. 4 and rearranging, we obtain

$$[LR] = \left( \frac{k_1}{k_2 + k_3} \right) [L][R] \tag{6}$$

Using a similar approach, we obtain from Eq. 5

$$[LRi] = \left( \frac{k_3 k_1}{(k_2 + k_3) k_4} \right) [L][R] \tag{7}$$

Assuming either that receptor degradation and synthesis are slow relative to the time scale of internalization, or that receptor synthesis balances any degradation, then the total receptor



concentration remains constant. Then, the sum of all receptor-containing species concentrations is equal to the total receptor concentration,

$$[R]_{TOT} = [R] + [LR] + [LRi] \quad (8)$$

Substituting Eqs. 6 and 7 into Eq. 8 and rearranging leads to

$$[R] = \frac{[R]_{TOT}}{1 + \left( \frac{k_3 k_1}{(k_2 + k_3) k_4} + \frac{k_1}{(k_2 + k_3)} \right) [L]} \quad (9)$$

We finally arrive at an expression for  $d[Li]/dt$  using Eqs. 7 and 9

$$\frac{d[Li]}{dt} = v_4 = \frac{k_3 \left( \frac{k_4}{k_3 + k_4} \right) [R]_{TOT} [L]}{\left( \frac{k_2 + k_3}{k_1} \right) \left( \frac{k_4}{k_3 + k_4} \right) + [L]} = \frac{V'_{max} [L]}{K'_m + [L]}, \quad (10)$$

where  $V'_{max}$  and  $K'_m$ , defined implicitly in Eq. 10, have analogous functional meaning as their enzyme kinetics counterparts. Eq. 10 thus predictably behaves as depicted in Fig. 2, with  $K'_m$  determining the ligand concentration at which the internalization rate is half-maximal, and  $V'_{max}$  determining the maximal internalization rate. Simulating the precise behavior of Eq. 10, however, requires values for  $V'_{max}$  and  $K'_m$ . These values are obtained via parameter estimation as described in the next section.

It is important to note that Eq. 10 is only valid given all of the assumptions made to derive this model, which may or may not apply. For instance, in many cases  $[L]$  is not much greater than  $[R]_{TOT}$ , and for some systems ligand binding may be rate limiting (Hendriks *et al*, 2006; Hendriks *et al*, 2003). It is a useful exercise to re-derive the internalization rate under such different conditions, which is left to the reader.

#### *Parameter Estimation: Determining Values of Unknown Model Parameters.*

Traditionally, to estimate  $V'_{max}$  and  $K'_m$ , standard enzyme kinetics practice, such as the use of the Lineweaver-Burk plot, would be applied. However, these methods rely on linearization of Eq. 10 by the substitution of the variables, which distorts experimental errors and can significantly bias parameter estimates. Therefore, a preferred method for estimating unknown parameters in such models is non-linear regression. Such a procedure works by changing parameter values to minimize the differences between Eq. 10 and experimental data. This procedure is sometimes referred to as “training” or “fitting”. Given data for how  $[Li]$  changes over time at various values of  $[L]$ , one readily can estimate  $V'_{max}$  and  $K'_m$  using non-linear regression. It can thus be said that  $V'_{max}$  and  $K'_m$  are *identifiable* from such a dataset. In the specific case of LDL in Hep-G2 cells, it was found that  $V'_{max} = 15.8$  ng LDL/mg cellular protein/min and  $K'_m = 48$   $\mu$ g/mL (Harwood *et al*, 1997).

In many instances, the specific rate constant  $k_3$ , also known as the endocytic rate constant  $k_e$  (Wiley and Cunningham, 1981, 1982), is of particular interest. Although the standard kinetics dataset is sufficient for estimating  $V'_{max}$  and  $K'_m$ , it is not sufficient for estimating  $k_3$ ; one must know  $k_4$  and  $[R]_{TOT}$  to estimate  $k_3$  from  $V'_{max}$ . The value of  $k_3$  is

therefore not identifiable from such a dataset, and no conclusions could be made based on its value. Below, an experimental protocol for estimating  $k_e$  will be discussed (Wiley *et al*, 1981, 1982).

**Model Validation: Comparing Model Predictions to Independent Experimental Data.** The final step of the modelling process, model validation, is to determine whether the model can *predict* rather than simply reflect system behaviour. There are few constraints on this step; a prediction can describe any property of the model behaviour, and experimental data can be any measured quantity. The only requirement is that the model was not fit to the experimental data being used for validation. The model in Eq. 10 was validated for LDL in Hep-G2 cells by comparing the predicted to measured internalization velocities (ng LDL/min/mg cell protein) and finding excellent agreement ( $R^2 = 0.965$ ) (Harwood *et al*, 1997). This phenomenological model can thus be considered valid for LDL in Hep-G2 cells. If the predictions did not agree with the data, one should return to Step 1 to propose a new model.

## Evolution of Mathematical Models of Endocytosis

In the early 1980's and 90's, only the basic, macroscopic steps of the endocytic and trafficking process, such as those shown in Fig. 1, were known. As such, models developed during these times consisted primarily of compartmental ODEs, which are able to describe the dynamics of species distribution in different cellular compartments. As our mechanistic understanding of endocytosis has grown, including discoveries of novel links between endocytosis and signalling, this new information has been increasingly incorporated into more and more complex mathematical models. Below we review how mathematical models of endocytosis have evolved over time as our biological knowledge has grown.

### Modeling Endocytosis and Signaling

**Pitfalls of Scatchard Analysis and the Endocytic Rate Constant.** Scatchard analysis is a general technique, which has been routinely used to calculate ligand affinities and receptor abundances and applied to studies of ligand trafficking. The dynamic nature of receptor endocytosis and trafficking, however, violates several assumptions underlying Scatchard analysis. In fact, depending on how fast receptor internalization is compared to ligand-receptor dissociation, one could obtain Scatchard plots showing negative, positive, or no cooperativity in ligand binding (Gex-Fabry and DeLisi, 1984). To circumvent these inherent problems with Scatchard analysis, a mathematical-modeling based approach for determining the endocytic rate constant  $k_e$  was developed (Wiley *et al*, 1981, 1982). The endocytic rate constant quantifies the rate at which ligand-bound receptors are internalized. The inverse of this rate constant is the average time that a single ligand-receptor complex remains on the cell surface before being endocytosed, or the half-life for this first-order process. We noted above that  $k_e$  was not identifiable from the data that describe the amount of internalized ligand vs. time. However, one can identify  $k_e$  by considering the dynamics of the initial  $[LRi]$  accumulation, provided that the two conditions apply. First, internalized ligand should remain receptor-bound over the time scale of observation, which gives  $d[LRi]/dt \approx v_3$ . Second, the concentration of surface ligand-receptor complexes should be essentially constant over the observation time scale, which means that  $[LR]$  is at pseudo steady-state. We denote this quantity as  $[LR]_{quasi-stationary}$ . We will follow the accumulation of  $[LRi]$  before the system reaches the steady-state, and, therefore, Eq. 7 above does not apply here. With these assumptions, we have

$$\frac{d[LRi]}{dt} = k_e [LR]_{quasi-stationary} \Rightarrow \frac{[LRi(t)]}{[LR]_{quasi-stationary}} = k_e t, \quad (11)$$

where  $t$  is time. Thus, one can identify the value of  $k_e$  as the slope of a straight line arising from a plot of the ratio of internalized to surface ligand-receptor complex concentrations versus time. Such data are readily attainable experimentally, and this relationship has been shown to hold in multiple systems (Wiley, 1988; Wiley *et al*, 1981, 1982).

For some systems, however, large ligand doses can lead to significant, rapid depletion of surface receptor numbers over the time scale of observation, inflating estimates for  $k_e$  and potentially violating the second condition above. For such cases, an alternative “internalization plot” method can be used to find  $k_e$  (Lund *et al*, 1990),

$$[LRi] = k_e \int_0^t [LR] dt, \quad (12)$$

which requires no additional data, but importantly does not rely on the pseudo steady-state of surface ligand-receptor complexes. The integration in Eq. 12 can be calculated from data using standard numerical quadrature methods, such as the trapezoidal or Simpson’s rules.

For the EGFR system, this modelling-based approach to measure  $k_e$  has resulted in the experimental observation that  $k_e$  decreases with increasing EGF concentration, rather than remaining constant, as in most receptor-mediated endocytosis systems (Wiley, 1988). Thus, for EGFR it appears that the higher the number of occupied surface receptors is, the longer the average occupied receptor stays on the cell surface. The explanation for this behaviour is that ligand-bound EGFR is simultaneously internalized via two pathways: one is rapid but has low capacity, while the other is slow but has high capacity (Lund *et al*, 1990; Wiley, 1988). This general system structure allows for internalization to switch from the low capacity route to the high capacity route in an ultrasensitive manner (Schmidt-Glenewinkel *et al*, 2008). The low and high capacity pathways have been identified as the clathrin-coated pit and constitutive caveolae pathways, respectively (reviewed in Sorkin *et al*, 2009). Ongoing studies focus on exploring the molecular links between EGFR signalling and clathrin-mediated endocytosis (CME).

*Compartmental models and governing principles of receptor-mediated endocytosis.* A variety of specific models have been developed to describe the endocytosis and trafficking processes for different ligand/receptor systems. Examples include the models for interferon and tumor necrosis factor in A549 cells (Bajzer *et al*, 1989), LDL in HepG2 cells (Harwood *et al*, 1997) and hepatocytes (Wattis *et al*, 2008), insulin/GLUT4 in adipocytes and 3T3-L1 cells (Holman *et al*, 1994; Yeh *et al*, 1995), Tf in MDCK cells (Sheff *et al*, 1999), and EGF in a variety of cell lines (Waters *et al*, 1990; Wiley, 1988; Yanai *et al*, 1991). These models, generally classified as compartmental ODE models, describe the dynamics of how ligands, receptors, and their complexes move into and out of various cellular compartments (Fig. 1). In such models, compartments are considered homogeneous, or “well-mixed”, and species concentrations within a single compartment are assumed constant, not varying with space.

While the compartmental ODE models for ligand/receptor endocytosis have helped generate specific knowledge about their respective systems, it has also become apparent that there are general governing principles applying similarly to a variety of receptor systems. Four of the classical endocytic systems, EGFR, TfR, LDLR, and VtgR (and possibly more) can all be described by the same simple model structure (Fig. 3) (Shankaran *et al*, 2007a; Shankaran *et al*, 2007b). This model structure is described by two key dimensionless parameters. The first is the partition coefficient ( $\beta = k_e/k_{off}$ ), which characterizes how fast

internalization (which is described by the endocytosis constant  $k_e$ ) is relative to ligand dissociation from the surface receptors ( $k_{off}$ ). The second parameter is the specific avidity ( $\gamma = (K_a R_{TS}) / (N_{av} V)$ , where  $R_{TS}$  is the total number of surface receptors,  $K_a = k_{on}/k_{off}$  is ligand affinity for the receptor,  $N_{av}$  is Avogadro's number, and  $V$  is the total system volume), which characterizes how effectively cells capture extracellular ligand. The values for these two parameters control the “relaxation time”, which determines how quickly the number of surface ligand-receptor complexes returns to steady-state after a standard impulse of external ligand (usually given as a very short pulse—mathematically characterized by a Dirac delta function). Although as both the partition coefficient and specific avidity increase, the relaxation time decreases, depending on the absolute values of these parameters, the relaxation time has different sensitivity to changes in their values. In fact, receptor systems can be classified into three basic regimes based on this differential sensitivity: (i) an avidity-controlled regime, in which the relaxation time is controlled by the specific avidity, but not by the partition coefficient, and where TfR and LDLR operate (ii) a consumption-controlled regime in which the relaxation time is sensitive to the partition coefficient but not to the specific avidity, and where VtgR operates and (iii) a dual sensitivity regime, in which both the specific avidity and the partition coefficient can effectively control relaxation time, and where EGFR operates. These results have biological implications, showing that the behaviour of different receptor systems can only be controlled in a certain way, depending on the regime. For TfR and LDLR, an effective strategy for control is changing the total surface receptor concentration  $R_{TS}$ . Indeed, cells upregulate the total levels of these receptors either at the transcriptional level or by rapid plasma membrane translocation from intracellular pools (Makar *et al*, 1998; Rao *et al*, 1986; Ward and Kaplan, 1986). Since TfR and LDLR are mainly involved in nutrient transport, it can biologically be preferable that their endocytic behaviour is primarily controlled by how effectively a cell can capture the nutrients. On the other hand, EGFR endocytosis can be controlled by either parameter, and is in fact regulated both by rapid ligand-induced internalization ( $\beta$ ) and degradation ( $\gamma$ ) (Lund *et al*, 1990; Wiley, 1988). On the other end of the spectrum, VtgR is only effectively controlled by the partition coefficient, and therefore is regulated by changes in  $k_e$  (Opresko and Wiley, 1987).

Densensitization and ligand/signal removal are widely accepted roles for ligand-induced receptor downregulation. However, given the emerging view that endocytosis plays major roles in signalling dynamics, one might expect that ligand-induced receptor downregulation can also control the signal processing characteristics of receptor systems. Indeed, as the rate of receptor downregulation increases ( $\beta$  increases), the relaxation time decreases and synchronization between the  $[L]$  and  $[LR]$  dynamics improves (Shankaran *et al*, 2007a; Shankaran *et al*, 2007b). Thus, ligand-induced receptor downregulation results in faster signal processing, which better reflects the extracellular ligand dynamics. There is a trade-off though, between increased response speed and output signal magnitude, which decreases with increasing downregulation. Such  $k_e$ -controlled response speed characteristics should be particularly important for chemotactic cell migration, where the number of ligand bound receptors must respond quickly to changing gradients of growth factors. Indeed, ligand-induced EGFR endocytosis seems to be critical for EGF-gradient induced cell migration (Caswell *et al*, 2008; Jekely *et al*, 2005). Importantly, such improved response speed behaviour only occurs for receptor systems, where the response speed is sensitive to changes in  $k_e$ . EGFR of course falls into the dual-sensitivity regime, which is effectively controlled by  $k_e$  (Shankaran *et al*, 2007a).

Interestingly, when ligand concentrations are small, as is typical of *in vivo* situations, the equations that govern the dynamics of the ligand-receptor system appear analogous to those that describe an automobile spring/shock absorber system (also known as a classical

harmonic oscillator or spring-dashpot system) (Shankaran *et al*, 2007b). In terms of the mathematical equations, the concentration of surface ligand-receptor complexes is analogous to the automobile vertical coordinate (position), the car mass is related to the total receptor concentration and ligand affinity, and external ligand concentration is analogous to an external force. Increasing receptor downregulation is the same as lubricating the shock absorber, which allows it to return to its proper position more quickly such that it is ready to respond to the next perturbation, being a change in ligand concentration or external force. Although too much lubrication (or too strong of a spring) may cause unwanted oscillatory behaviour in the car, it was predicted that the EGFR system will never display such oscillatory behaviour no matter how large  $k_e$  becomes (Shankaran *et al*, 2007b). This modelling-based prediction has yet to be tested experimentally.

We conclude that despite the diversity of receptor-mediated endocytosis systems, important features of their temporal behaviour can be characterized by only two dimensionless parameters.

*Incorporating signalling processes into models of receptor trafficking.* The notion that endocytosis and signal transduction are two inseparable, bi-directionally interacting systems is becoming increasingly appreciated. We first will focus on signalling and endocytosis of the ErbB growth factor receptor family, which operates in multiple tissues and whose deregulation is implicated in the development and progression of several types of cancer (Yarden and Slivkowski, 2001). The ErbB family consists of four receptors, ErbB1, 2, 3 and 4, and ErbB1 is also known as EGFR. When ligands bind to these receptors, the receptors can homo and heterodimerize with each other; this activates their intrinsic tyrosine kinase activity and propagates signals into the cell. Although different ligands target distinct ErbB family receptors, downstream signalling is funnelled through shared kinase cascades, such as the extracellular regulated kinase 1/2 (ERK) cascade and the phosphatidylinositol 3-kinase PI3K/AKT pathways. The distinct temporal profiles and duration of ERK1/2 activation were shown to be the determinant of critical cell-fate decisions, such as differentiation versus proliferation (Kholodenko, 2007; Marshall, 1995). For example, EGF or heregulin (HRG) induce transient vs sustained activation of ERK and proliferation vs differentiation of MCF7 breast cancer cells (Nagashima *et al*, 2007). It was shown both experimentally and using simulations *in silico* that the pronounced difference in the temporal profiles of effector kinases activated by distinct receptors is related, at least partially, to the different kinetics of receptor endocytosis (Birtwistle *et al*, 2007; Haslekas *et al*, 2005; Hendriks *et al*, 2005; Lynch *et al*, 2004). This is due to the fact that only EGFR, and not ErbB2-4, undergo significant ligand-induced endocytosis and degradation. While both EGFR homodimers and EGFR-ErbB2 heterodimers are rapidly internalized, EGFR-ErbB2 homodimers recycle to the cell surface, whereas EGFR homodimers remain internalized. Thus, ErbB2 overexpression, which occurs in ~25% of all breast cancers, preserves EGF receptors at the cell surface, preventing EGF and EGFR degradation and sustaining mitogenic signalling.

Endocytosis and trafficking has been important to consider for proper modelling of ErbB signalling (Birtwistle *et al*, 2007; Resat *et al*, 2003; Schoeberl *et al*, 2002), and also for modelling of other systems, such as transforming growth factor  $\beta$  (TGF $\beta$ ) (Vilar *et al*, 2006), platelet derived growth factor (PDGF) (Wang *et al*, 2009) and EGF/insulin co-induced signalling (Borisov *et al*, 2009). Although all these studies incorporate significant molecular detail of signal transduction processes, they predominantly describe trafficking processes as they were known in the 1980's, i.e., without the mechanistic detail that is now becoming available. Despite the mechanistic simplicity of endocytosis descriptions within complex signal transduction models, the simulation results made it clear that endocytic processes play a major role in dictating the outcome behaviour. Future signal transduction models will incorporate the molecular mechanisms of endocytic processes to understand their precise

roles in the regulation of signal transduction, as well as their potential implications for cancer studies.

### ***Modeling Endocytosis in Space and Time***

So far, we focused on the temporal aspects of endocytosis, while the spatial aspects of signal transfer were only roughly accounted for by considering well-mixed interacting compartments at different locations. Although such approaches are valuable, many aspects of endocytosis and signalling can only be understood by considering the spatial dimension explicitly. Mathematically, this involves PDEs, which naturally arise from the description of rate processes in both space and time.

*Brief basics of spatial modelling.* The derivation of a PDE model is conceptually similar to that of an ODE model: a general species balance equation is applied that sets the temporal rate of change of a species *in a particular spatial location* equal the sum of all processes that produce the species, minus all those that consume it, *plus the net species movement rate into that location*. This verbal definition can be summarized mathematically as

$$\frac{\partial C(x,y,z,t)}{\partial t} = \sum_i v_{pi} - \sum_i v_{ci} + v_m. \quad (13)$$

Here, the left hand side is the partial derivative of species concentration  $C$  with respect to time  $t$ , the dependence of  $C$  on both time and the spatial coordinates  $x$ ,  $y$ , and  $z$  is denoted explicitly,  $\Sigma$  denotes a summation, and  $v$  denote rates, with subscripts  $p$  for production,  $c$  for consumption, and  $m$  for movement. Within cells, the net movement rate  $v_m$  of species molecules can be subdivided into a diffusive component  $v_d$  and trafficking component  $v_t$ . Assuming that the diffusivity  $D$  of species  $C$  does not change in space, the diffusive transport rate can be described by

$$v_d = D\Delta C = D\left(\frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2}\right), \quad (14)$$

where  $\Delta$  is the Laplacian operator, whose expression is given for a standard Cartesian, or rectangular, coordinate system. Trafficking is velocity-driven transport, when all molecules move with the same particular velocity at each location. Therefore, the trafficking transport rate depends on the directional velocities, which we denote by  $V_q$ , where  $q$  is  $x$ ,  $y$ , or  $z$ . If the concentration  $C$  would not change in space, then directional trafficking of molecules of the species  $C$  would not change the amount of molecules in any particular location. If the concentration is decreasing in the direction of positive velocity, then velocity-driven transport increases the concentration, and vice versa. Whether concentration is decreasing or increasing is characterized by the concentration gradients, or the first partial derivatives of concentration with respect to the spatial dimensions. Thus, the trafficking transport rate can be written as,

$$v_t = -\left(V_x(x,y,z,t)\frac{\partial C}{\partial x} + V_y(x,y,z,t)\frac{\partial C}{\partial y} + V_z(x,y,z,t)\frac{\partial C}{\partial z}\right). \quad (15)$$

Although in Eq. 15, the velocities can change in space and time, this equation is predicated upon the assumption that these velocities change continuously and smoothly. Thus, these convection PDE models cannot capture the stochastic, “back and forth” movement of endosomes (Wacker *et al.*, 1997). However, stochastic fluctuations can be less important for bulk species transport over the long distances ( $\mu\text{m}$ ) and time scales (minutes).

Putting everything together we arrive at Eq. 16, which is commonly referred to as a reaction-diffusion-convection equation (for a standard Cartesian coordinate system).

$$\frac{\partial C}{\partial t} = \sum_i v_{pi} - \sum_i v_{ci} + D \left( \frac{\partial^2 C}{\partial x^2} + \frac{\partial^2 C}{\partial y^2} + \frac{\partial^2 C}{\partial z^2} \right) - \left( V_x \frac{\partial C}{\partial x} + V_y \frac{\partial C}{\partial y} + V_z \frac{\partial C}{\partial z} \right) \quad (16)$$

Modelling of endocytosis thus far has primarily considered the reaction and diffusion components, although there is no real barrier to including the trafficking components.

*Endocytosis and spatial signalling gradients.* In many signal transduction systems, signals originate on the plasma membrane, but should propagate to distant locations, such as the nucleus. For example, EGFR generates phosphorylation signals that propagate through the membrane-bound Ras to the ERK pathway, and active, phosphorylated ERK travels to the nucleus to enact gene expression changes. If signal deactivators (terminators), such as phosphatases, are homogeneously distributed in the cell and signals can only move by diffusion, then as one moves away from the plasma membrane, there will be fewer signals, creating a signalling gradient. Without endocytosis, the depth of such a gradient depends on the signal deactivation rate and the diffusion coefficient. If signal deactivation is a first order process with rate constant  $k$ , then the level of active signal exponentially decays away from the plasma membrane, with characteristic length  $\alpha^{-1} = \sqrt{k/D}$  (Brown and Kholodenko, 1999). Thus, if signal deactivation is fast, or diffusion is slow, then precipitous signalling gradients may occur. In fact, a typical deactivation rate constant of  $\sim 1 \text{ s}^{-1}$  and protein diffusion coefficient of  $\sim 1 \mu\text{m}^2 \text{ s}^{-1}$ , gives  $\alpha^{-1} \sim 1 \mu\text{m}$ , much smaller than the distance from the plasma membrane to the nucleus in large mammalian cells. Since endocytosis brings signals from the plasma membrane into the cell interior, closer to the nucleus, one plausible role of endocytosis may be to overcome this problem of potentially steep activity gradients that can impede spatial signal propagation. This situation may be represented as a simple reaction-diffusion model, schematically shown in Fig. 4A (Kholodenko and Birtwistle, In Press), and described by,

$$\frac{\partial C}{\partial t} = 0 = D\Delta C - kC \quad (17)$$

where  $\Delta$  here is the Laplacian operator in spherical coordinates (different from rectangular coordinates). Since endosomes move slowly compared to the time scale of signalling processes, we assume the position of endosomes to be fixed and located halfway from the plasma membrane to the nucleus for illustrative purposes. If half of the signal generators are in the signalling endosomes, while the other half reside on the plasma membrane of a cell with radius  $R_{\text{cell}}$ , the steady-state concentration gradients behave as shown in Fig. 4B-C. If  $\alpha^* R_{\text{cell}} \gg 10$ , then either diffusion is too slow or deactivation is too fast for endocytosis to help signal propagation. On the other hand, if  $\alpha^* R_{\text{cell}} \ll 10$ , then signals can propagate without help from endocytosis. When  $\alpha^* R_{\text{cell}} \sim 10$ , endocytosis may play a critical role in spatial signal propagation from the plasma membrane to the cell nucleus (Kholodenko and Birtwistle, In Press).

*Long-distance transport.* Spatial gradients pose a particular problem when signals travel over distances greater than 100  $\mu\text{m}$ , where diffusive transport is insufficient, because of the following: (i) diffusion is too slow, since the distance traveled by diffusion is proportional to the square root of time and (ii) signals commonly vanish owing to termination by deactivating enzymes in the media. For the transport of signals from the plasma membrane to the nucleus in large cells like *Xenopus* oocytes ( $\sim 1$  mm diameter), based on the above  $\alpha \cdot R_{\text{cell}} \sim 10$  condition, if signal deactivation (termination) activity is low ( $\alpha < 0.01 \mu\text{m}^{-1}$ ), then endocytosis combined with simple diffusion is a plausible mechanism for signal propagation to the nucleus in such cells. However, typical diffusion coefficients ( $\sim 1\text{-}10 \mu\text{m}^2/\text{s}$ ) and termination activities ( $\sim 1 \text{ s}^{-1}$ ) give  $\alpha \sim 1\text{-}0.1$ , so unless termination activity is regulated to be extremely low during the initial time period of 10-20 minutes following stimulation, it is unlikely that endocytosis plays a significant role for signal propagation in *Xenopus* oocytes. Alternatively, cytoplasmic scaffolds and molecular-motor driven transport of signaling complexes may play a role in spatial signal propagation by protecting signals from cytoplasmic dephosphorylation (Perlson *et al*, 2005; Perlson *et al*, 2006).

Centimeter and even meter scale transport of signals, such as from neuron terminals to the nucleus via the axon (e.g. from a giraffe's lower leg to its brain), present an even more challenging problem for signal propagation within a cell (Howe *et al*, 2004). Although the retrograde transport of endosomes is an important signaling vehicle, in the NGF-TrkA system, signals can propagate through mechanisms other than endosomal transport (MacInnis *et al*, 2002). Additionally, the average velocity of molecular motors ( $1\text{-}10 \mu\text{m}/\text{sec}$  (Hill *et al*, 2004)) is not fast enough to account for experimentally observed signal propagation time (MacInnis *et al*, 2003), posing the question of what mechanisms may be able to transport signals faster than retrograde transport, and over distances of meters. It has been proposed that traveling waves of protein activation can perform this task (Markevich *et al*, 2006). Such waves can occur when a downstream kinase positively feeds back to a cytoplasmic upstream kinase, and the stimulus duration exceeds a certain threshold. Simulation results suggest that these traveling waves transport signals at tens of  $\mu\text{m}/\text{sec}$ , and as the strength of positive feedback is increased, the velocity increases (up to hundreds of  $\mu\text{m}/\text{sec}$ ). These traveling waves are much faster than retrograde transport; fast enough potentially to explain the experimentally observed speed of signal propagation in the NGF-TrkA system (Markevich *et al*, 2006).

*Endocytosis and cell polarization.* Not only is endocytosis closely related to signal transduction, but it also plays a key role in yeast cell polarization, which is manifested by highly localized Cdc42 concentration (Marco *et al*, 2007; Valdez-Taubas and Pelham, 2003). Modelling suggests that a main cell polarization mechanism involves localized endo- and exocytosis in a small region of the plasma membrane called a "directed transport window", coupled with characteristically slow diffusion of proteins in the yeast plasma membrane (Marco *et al*, 2007; Valdez-Taubas and Pelham, 2003). In fact, any *in silico* changes to the measured local endocytosis rates reduced the model-predicted height and sharpness of the local Cdc42 concentration peak, or polarity, thus suggesting the specific endocytosis rate magnitudes have evolved to optimize cell polarity. However, protein diffusion in the yeast plasma membrane is much slower than that in a mammalian cell plasma membrane, and it is yet unclear whether the same polarization mechanisms and optimality apply in mammalian systems.

Endocytosis can also play a role in creating non-uniform distributions of receptors on the cell plasma membrane. When giant Hela cells are evenly spread on a surface, there is a non-uniform distribution of Tf and LDL receptors, which are concentrated at the periphery



(Bretscher, 1983). However, since coated pits are uniformly distributed on the cell surface, endocytosis develops homogeneously. On the other hand, exocytosis occurs only at the leading edge of Hela cells (Bretscher, 1983). Based on these considerations, a PDE model was developed to explain this non-uniform surface receptor distribution, which consists of a description of both bulk membrane trafficking and receptor transport on a disc-shaped cell (Goldstein and Wiegel, 1988). Since endocytosis occurs uniformly over the entire cell surface, but exocytosis only replaces membrane at the leading edge, there is bulk membrane flow from the leading edge to the cell centre. Modelling suggests that the bulk membrane velocity  $v$  is linearly proportional to the distance from the cell centre ( $r$ ) and inversely proportional to the mean time ( $\tau_m$ ) that a uniformly distributed membrane component spends on the cell surface ( $v = -r/2\tau_m$ ). In this scenario, the membrane velocity is largest at the periphery and zero at the cell centre. As a result, any receptor on this moving membrane will have a non-uniform distribution, simply because receptors flow away from the cell periphery faster than they do from interior points. However, the distribution of receptors depends not only on the membrane velocities but also on diffusion and the kinetics of receptor endocytosis. At the cell periphery where membrane velocity is high, diffusion plays a small role for the receptor distribution. Since velocity decreases as the cell centre is approached, there is a critical radius  $r^* = (4D\tau_m)^{1/2}$  where the membrane flow velocity equals effective receptor diffusive velocity. At this radius, membrane velocity and diffusion have equal control over the receptor distribution, and points interior to this radius are diffusion controlled. Thus, as the diffusion coefficient increases, a greater percentage of the receptor distribution on the cell surface is diffusion-controlled, and therefore more uniform. In terms of endocytosis, if receptors are excluded from coated pits, then they are endocytosed more slowly than a uniformly distributed membrane component. In this case, membrane flow and diffusion dominate the receptor transport, which causes receptors to localize towards the cell centre. On the other hand, if receptors are preferentially recruited to coated pits, making their endocytic rate faster than that of a uniformly distributed component, then endocytosis dominates the receptor transport, and more receptors are located towards the cell periphery. Thus, changing global receptor endocytosis rates may create qualitatively different distributions of receptors on a cell surface.

*Endocytosis and cancer treatment efficacy.* The success of cancer treatments depends on the spatial distribution of the treatment within the tumor. Although small molecule chemotherapeutics freely diffuse into cells, many large treatments, such as monoclonal antibodies, toxin-conjugated antibodies, and enzyme-conjugated antibodies, bind to and are endocytosed by tumor cells. When these cures are endocytosed, they remain inside the cell to be eventually degraded, but importantly are not able to penetrate further into the tumor. Thus, endocytosis affects the spatial distribution of cures within a tumor, and therefore their efficacy. To understand the precise roles endocytosis plays in determining cancer treatment efficacy, spatial reaction-diffusion models have been developed. (Ackerman *et al*, 2008; Dee and Shuler, 1997; Thurber and Wittrup, 2008; Roth, 2005; Zhu *et al*, 2006). For treatments whose action only requires cell surface binding, as endocytic rates increase drug penetration and therefore efficacy decrease; models in this case can provide design criteria for upper bounds on endocytic rates of the treatment. On the other hand, for drugs whose action is improved and/or depends upon internalization, there is an optimal endocytic rate for drug efficacy, since faster endocytosis would lead to more medication inside tumor cells, but at the cost of less spatial penetration into a tumor. For these treatments, reaction-diffusion models can be used to find this optimum endocytic rate. Thus, not only can spatial models help us understand the biochemistry and cell biology of endocytosis, but can also play an important role for guiding cancer treatment design.

## **Future Directions**

Modelling of endocytic processes has clearly provided insight into their dynamic behaviour that would otherwise not be possible. Modelling will continue to play a role, as biological research uncovers new mechanistic knowledge and raises new questions about endocytosis and signalling. More mechanistic descriptions of endocytosis and trafficking processes will allow us to gain insight into how specific proteins, such as dynamin, clathrin, epsin, AP-2, Cbl and the Rab family GTPases, precisely control trafficking rates and protein distributions. Another major frontier is full-fledged integration of signalling and endocytic processes. Molecular knowledge on the signalling level is vast and has been heavily incorporated into models. While endocytic processes have been incorporated into many signal transduction models, their description has been mostly phenomenological, not considering the rich mechanistic dialogue between signalling and endocytosis. New mechanistic models will allow us to address yet unresolved questions, such as what is the exact contribution of signalling on endosomes to the overall signalling responses. Importantly, these models will also allow us to uncover roles for the newly found molecular mechanisms and links between endocytosis and signalling.

## Figure Legends

**Figure 1. A general, simplified scheme of endocytic and trafficking processes.** A small area of the plasma membrane invaginates (stage 1), and then it is pinched off into a vesicle (stage 2). The vesicle lumen contains previously extracellular material (including ligands  $L$  (shown by red) bound to the receptor ( $R$ ) extracellular domains), while the vesicle surface that faces the cytoplasm contains the material, previously exposed into cytosol (including the cytoplasmic receptor tails (shown by yellow) with bound adaptor or target proteins (shown by green)). The internalized vesicle fuses with larger, early endosomes. The internalized material is then trafficked to tubulovesicular sorting endosomes. From here, material can either be taken to recycling endosomes and thus back to the cell surface, or be re-internalized, creating a multi-vesicular body destined for lysosomal degradation.

**Figure 2. Relationship between the rate of internalized ligand accumulation and extracellular ligand concentration.** The rate of internalized ligand accumulation,  $v_4$ , is plotted against the concentration of extracellular ligand  $[L]$ . Importantly, this behavior is predicated upon Eq. 10 and all the assumptions used to derive it, and may not be applicable to other situations.

**Figure 3. A compartmental, "in silico" view of endocytosis and trafficking.** Extracellular ligand  $L$  binds to receptor  $R$  with rate constant  $k_{on}$  and the receptor-ligand complex  $LR$  dissociates with rate constant  $k_{off}$ . Empty receptors are internalized with rate constant  $k_i$ , whereas ligand bound receptors are internalized with rate constant  $k_e$ .

**Figure 4. Effects of endocytosis on spatial signal propagation. A.** Schematic of endocytosis-enhanced signal propagation in a spherically symmetric cell. Phosphorylation signals are generated at the plasma membrane and in a small region of space where the endosomes reside, and are terminated by phosphatases everywhere else. Signals can diffuse throughout the region between the plasma and nuclear membranes. **B and C.** Steady-state spatial profiles of the concentration  $C$  of active signal as a function of the distance  $d$  from the plasma membrane  $d=R_{PM}-r$ , where  $R_{PM}$  is the cell radius. The signaling endosomes are assumed to be loosely packed in the cylindrical layer between  $R_{endos}^{PM}$  and  $R_{endos}^{NM}$ . Dash-dot lines correspond to cases where half the kinase activity localized at the endosomes, while solid lines denote cases where all kinase activity is located at the plasma membrane. Panel B corresponds to a cell with dimensions  $R_{PM}=9\text{ }\mu\text{m}$ ,  $R_{nuc}=3.5\text{ }\mu\text{m}$ ,  $R_{endos}^{PM}=6.5\text{ }\mu\text{m}$ ,  $R_{endos}^{NM}=6.2\text{ }\mu\text{m}$ . The endosome width,  $0.3\text{ }\mu\text{m}=R_{endos}^{PM}-R_{endos}^{NM}$ , was taken to be three times a typical endosome diameter ( $\sim 100\text{ nm}$ ), since endosomes are not well-packed. Panel C corresponds to a large cell, such as a *Xenopus* oocyte, with dimensions  $R_{PM}=1000\text{ }\mu\text{m}$ ,  $R_{nuc}=390\text{ }\mu\text{m}$ ,  $R_{endos}^{PM}=650\text{ }\mu\text{m}$ , and  $R_{endos}^{NM}=649\text{ }\mu\text{m}$ .

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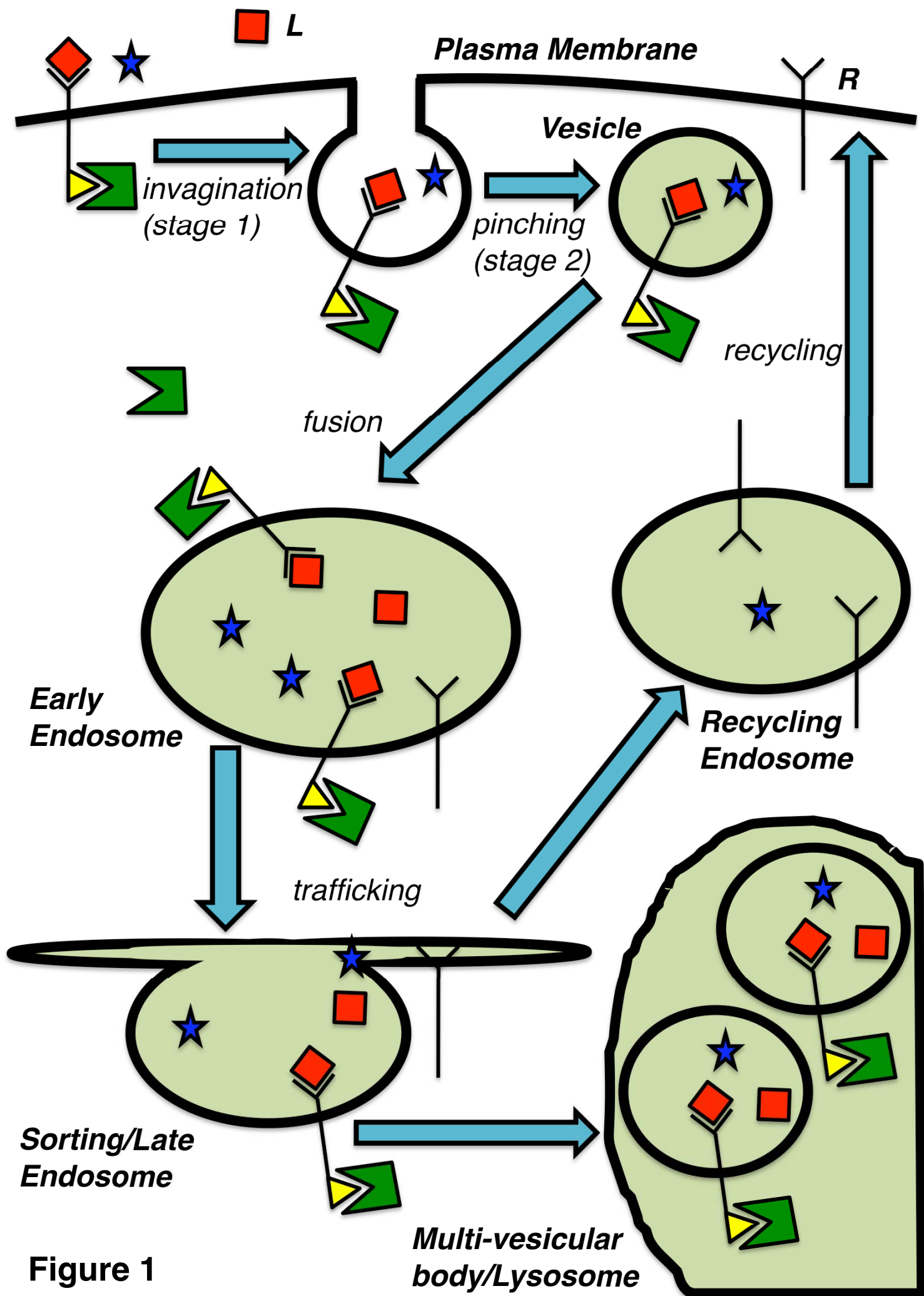
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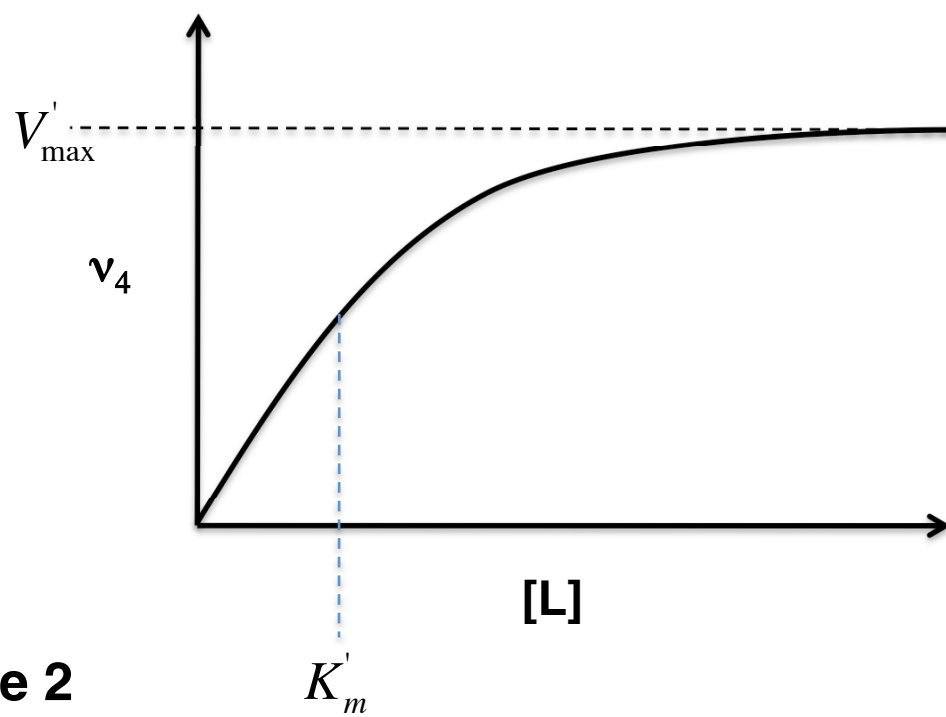
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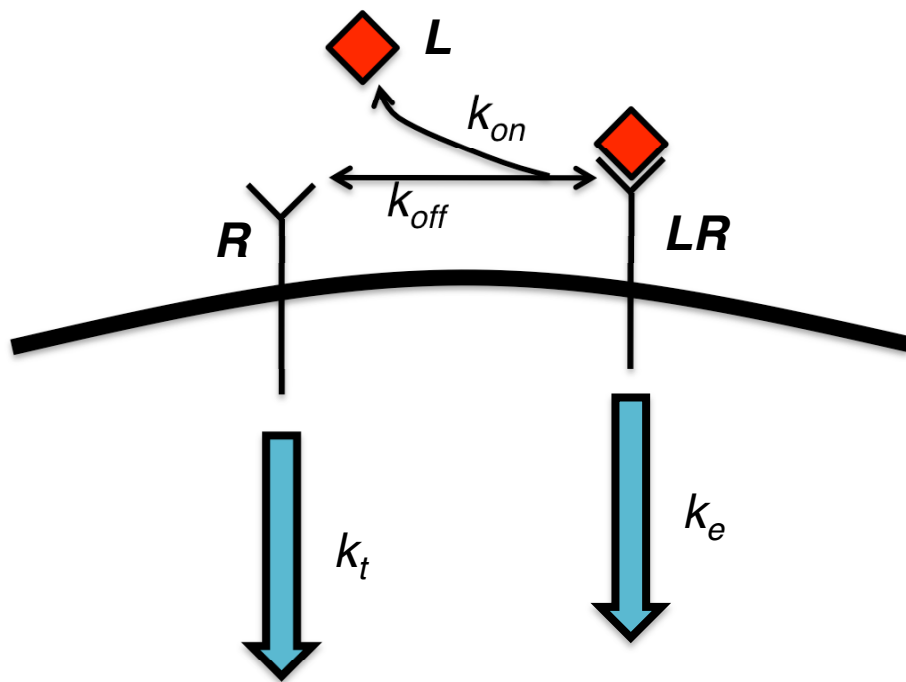
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**Figure 1**

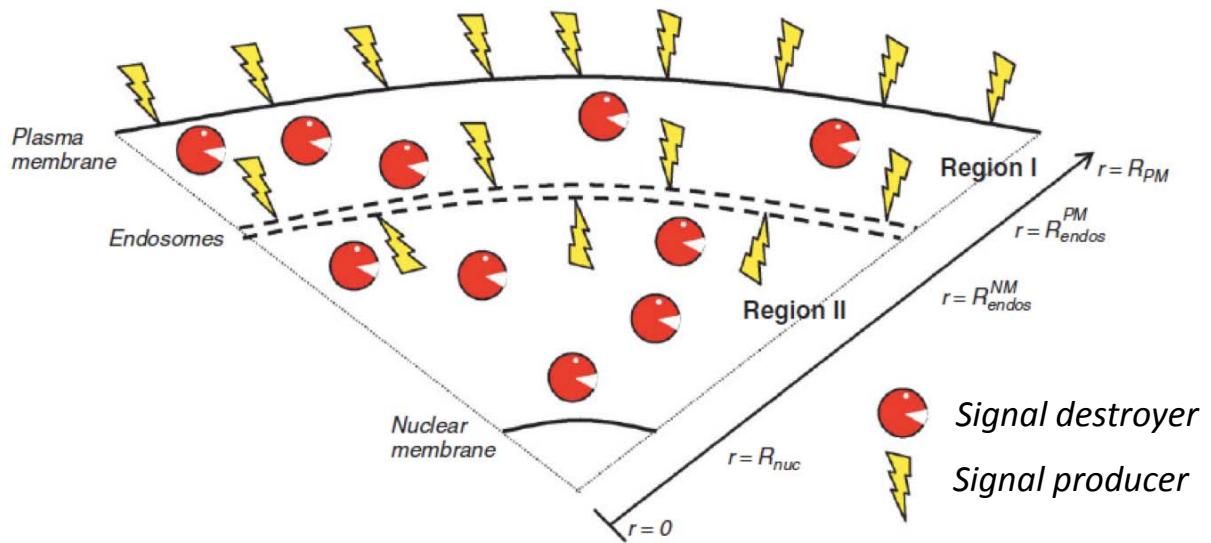


**Figure 2**

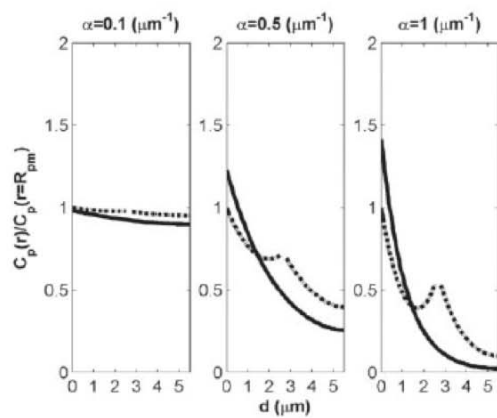


**Figure 3**

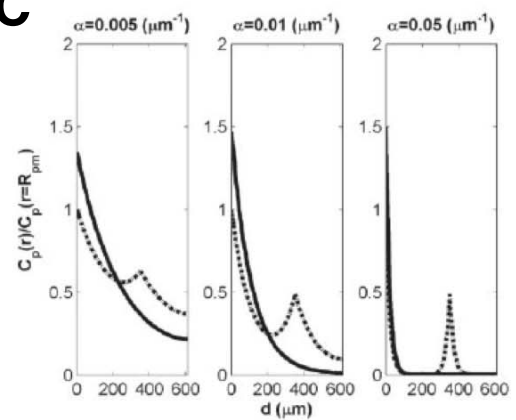
**A**



**B**



**C**



**Figure 4**