## Acknowledgments

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## Data availability statement

No new data were created or analysed in this study.

## Conflict of interest

The authors declare that they have no competing financial interests.

## 4. Extracellular signals and dynamic intracellular change

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### 4.1. Status

Cells have been selected for change. Even microbes use current signals to prepare for the future [21], and in our own cells circadian rhythms have hard-coded such preparation into a daily occurrence. In natural environments, be that a human tissue or as part of a microbiome, extracellular signals are likely to be multifarious, simultaneous, and continually varying. Yet it is only recently that microfluidic technology has allowed us to overcome the technical challenge of mimicking such signals.

Signaling networks should perform best in natural environments, and using dynamic inputs is proving a powerful means to understand their internal logic [22] (figure 2). There are mutants in the signaling pathway responding to hyperosmotic stress in budding yeast that only become distinguishable from wild-type when exposed to time-varying inputs [23], and some stress responses in bacteria respond not only to stress but also to its rate of increase [24]. Higher organisms may even regulate extracellular environments to become dynamic and use oscillatory levels of cytokines to selectively entrain signaling pathways [25].

Intracellular responses are dynamic too, and even a step change in an extracellular concentration can generate complex intracellular behavior. The levels of second messengers, such as calcium and cAMP, can spike or oscillate; metabolic cycles might change phase; and some transcription factors pulse in and out of the nucleus.

We are only beginning to understand why cells might use such dynamic signaling over steady-state responses. Dynamic responses are potentially quicker than waiting for steady-state behavior and also may carry more information because not only the amplitude but also the timing of the response can be used [26, 27]. Signaling pathways at steady-state appear to encode only enough information to distinguish between two types of environment, but the information substantially increases if the downstream biochemistry can sense the response's dynamics. Encoding different extracellular signals in the dynamics of signaling molecules can also coordinate downstream responses. A transcription factor that pulses in and out of the nucleus with a frequency but not amplitude that changes in different environments will always have the same concentration when in the nucleus, causing all regulated genes to respond together [28].

### 4.2. Current and future challenges

Characterising dynamic behavior requires finding suitable reporters. Their quality constrains the time resolution, the numbers of cells monitored, and the numbers of variables measured. Reporters must respond on appropriate time scales to capture dynamics, be sensitive to short acquisition times, and sufficiently responsive to excitation to limit photo-toxicity. Although monitoring transcription using RNA-binding proteins and signal transduction through nuclear translocation are both fast, each can potentially perturb intracellular dynamics.

A second challenge is choosing the input. Typically, we do not know the natural signals under which cells have evolved, if the input should change with time, or if it should appear alone or co-vary with others. A dynamic input greatly increases the number of variables—up to one for each time point. Exploring such a vast space is daunting, and without efficient methods we must make do with low sampling.

Studying individual cells itself raises problems because cellular context can determine behavior. As well as the inherent stochasticity of biochemistry, cellular history—how cells were prepared and previous exposure to signals—and cell state, such as phases of the cell cycle, metabolic cycle, or circadian cycle, can alter responses and confound interpretation. To make matters worse, we often do not have reporters for such endogenous rhythms. This variation means that we need quantitative methods to compare collections of time series. For example, there is no standard procedure to determine statistically significant differences between two sets of time series, such as for a wild-type and mutant.

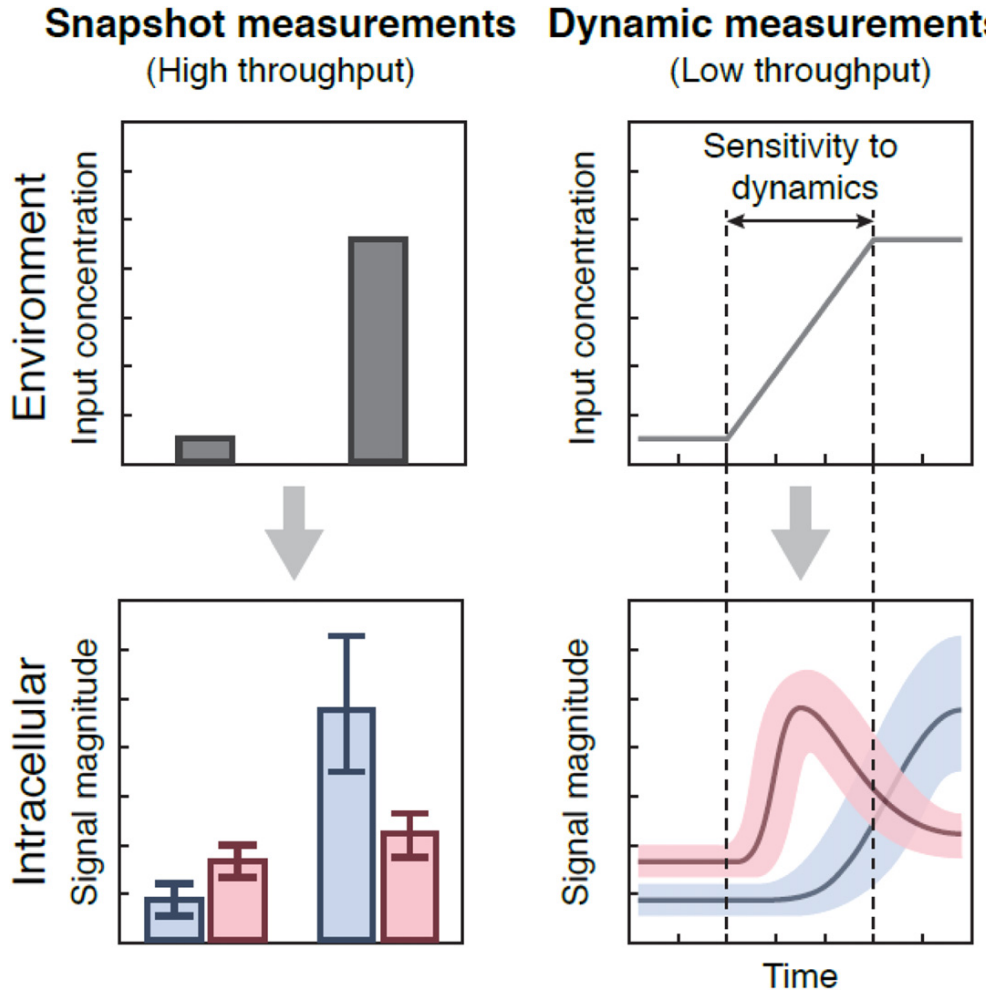


**Figure 2.** Some cellular responses are only revealed by dynamic inputs. In steady-state experiments, the blue but not the red molecule appears to respond to the input. A dynamic input, however, shows that the red molecule responds as strongly as the blue molecule, but to the input's time-derivative not its absolute value.

Although microfluidic technology has become indispensable, the device's design could bias intracellular dynamics. Often a device favors particular cellular shapes, and being confined can stress cells and alter gene expression. As the experiment runs, the cells under study can become unrepresentative of natural populations. For example, multiple devices trap cells but allow offspring to escape, and imaging for say eight generations means that the trapped cells constitute only $2^{-8}$ of a growing population. Further, polydimethylsiloxane (PDMS), the polymer often used in devices, can influence cellular behavior and absorbs hydrophobic molecules, potentially distorting inputs.

### 4.3. Advances in science and technology to meet challenges

Better reporters of intracellular activity would be transformative. Cross-talk between fluorophores limits most studies to two reporters, giving only a blinkered view of the response. Although we can control some signaling, such as kinases made sensitive to 1-NM-PP1 and through targeted degradation and optogenetics [29], we cannot measure *in vivo* the drivers of cellular decision-making—active kinases and phosphatases. Non-perturbative methods to follow RNAs and cellular cycles as well as reporters to quantify cellular context—levels of cofactors like $NAD^+$, of second messengers, and of energy (the ATP to ADP ratio, proton motive force, and membrane potential)—are all essential.

To mimic natural environments, we need reproducible control of the dynamics of inputs, the ability to apply multiple inputs, both simultaneously and sequentially, and optimization to efficiently explore the space of inputs. Chemical methods to reduce the hydrophobicity of PDMS, like silanization, will both prevent microfluidic devices perturbing inputs and enable new dyes as intracellular reporters.

Progress is needed on two bottlenecks: extracting information from time-lapse experiments and efficient means to search and share time-lapse data. Many laboratories develop in-house software for phenotyping cells that is too customized for data from elsewhere, and results must often be manually

corrected. Advances in convolutional neural networks should fix both problems. With sufficient training data, these algorithms work better and faster than traditional approaches, and techniques for transfer learning are facilitating sharing [30]. Agreeing on a standard format for storing images, annotations and associated meta-data will allow both exchanges and the meta-analyses needed for 'whole-cell' modeling.

Perhaps the most impact will be from combining time-series experiments with single-cell'omics. If a group of cells that has displayed a particular dynamic phenotype could be selectively extracted from a microfluidic device, then single-cell transcriptomics and proteomics will give numbers of reporters impossible to achieve with fluorescence, albeit at one time point. We will then be able to determine how the dynamics of inputs, movements of transcription factors, individual cell physiologies, and phases of endogenous rhythms in the recent past affect current programmes of gene expression.

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## 5. The role of timing in biological perception and actuation

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### 5.1. Status

Physicists often study biological networks as closed systems that evolve according to their own autonomous nonlinear dynamics. For example, a cell observed under the microscope will reliably move through stages of growth, DNA replication, and mitosis, taking an observer through each step of the cell cycle. Such a system lends itself well to modeling, and various emergent properties can be predicted: the stable states the cell finds itself in for extended periods of time, the speed at which it moves from state to state, and the period of the cycle.

But our picture of the cell cycle as a closed system is incomplete, as cell growth and division are highly responsive to environmental cues: local cell density, nutrient availability, the presence of permissive growth factors, and even subtle variations in temperature that elicit a biological stress response can dramatically alter or halt cell cycle progression.

It may thus be more fruitful to view the cell not as a closed, autonomous system but as a set of signal processing devices. We may borrow concepts from information theory, circuit design, and control theory to ask what dynamic filters, relays, and data compressors may underlie the cell's response to environmental cues [31]. How are useful and pertinent signals deciphered from a sea of external chemical and mechanical cues? Is there a 'code book' for intracellular signal transmission?

### 5.2. Current and future challenges

A few key biological processes are emerging as ideal context for studying cellular signal processing (figure 3). One is embryo development, where almost every transition is closely linked to a biological clock or timer. After all, cells have a limited time to migrate, divide or differentiate before the embryo proceeds to its next developmental stage.

For example, the *Drosophila* embryo's first fourteen nuclear cycles occur under extremely stereotyped time intervals and after approximately 3 h culminate in the profound cell movements associated with gastrulation. The formation of the segmented body plan and three germ layers must be completed on this timeline, requiring fast (minutes–hours) signaling events and transcriptional responses. Supporting this view, we found that Erk-dependent differentiation into gut endoderm and neural ectoderm was limited to a critical time window between nuclear cycle 10 (when nuclei move to the embryo's surface and can receive Erk-dependent signaling) and gastrulation [32]. The total duration of Erk signaling delivered in this narrow, 90 min time window proved to be essential for cell fate specification.

A second key context for dynamic signaling can be found in the maintenance, homeostasis, and repair of adult tissues. Numerous signaling pathways that were crucial to embryo development are again repurposed in the adult organism, where the objective is not the timely progression through embryogenesis but rather continuous tissue- and organism-level homeostasis.

Yet despite a high degree of molecular conservation, the requirements for homeostatic signaling are quite different than those in development. Homeostatic signaling must be sensitive, detecting a single defective cell among millions of normal ones; in contrast, inductive developmental cues can be produced at high concentrations. Homeostatic pathways must also respond to inputs with a huge range of unpredictable spatial distributions and timescales (e.g., wounds can be tiny or huge, acute or chronic), whereas developmental cues usually occur in predictable time windows. A sophisticated degree of information processing is essential to meet these varied constraints.

Interestingly, mounting evidence suggests that cell signaling in adult tissues also possesses its own complex spatiotemporal behavior [33]. This may include pulses of pathway activity, traveling waves across a tissue field, or switch-like and irreversible cell–fate transitions (e.g., apoptosis). In some cases, it is not obvious which stimuli are responsible for the observed the