Trends in Biotechnology

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Review

Accelerating Live Single-Cell Signalling Studies

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The dynamics of signalling networks that couple environmental conditions with cellular behaviour can now be characterised in exquisite detail using live single-cell imaging experiments. Recent improvements in our abilities to introduce fluorescent sensors into cells, coupled with advances in pipelines for quantifying and extracting single-cell data, mean that high-throughput systematic analyses of signalling dynamics are becoming possible. In this review, we consider current technologies that are driving progress in the scale and range of such studies. Moreover, we discuss novel approaches that are allowing us to explore how pathways respond to changes in inputs and even predict the fate of a cell based upon its signalling history and state.

Trends

Major advances in the speed, and ease, with which we can tag proteins, and design reporters for their activity, are driving a surge in live single-cell studies.

The throughput of live single-cell studies is currently limited by challenges in tracking and data extraction. Adopting approaches used in multiple object tracking has led to great progress in this area.

Live single-cell studies coupled with a range of technologies are allowing us to probe signalling networks, shedding new light upon the dynamics of how cells process information and undertake decisions regarding their fate.

Live Single-Cell Imaging Leads the Way

In response to changing environmental conditions, cells often veer from equilibrium and undertake systems-wide changes to promote homeostasis at the cell, tissue, and organism levels. These changes include decisions to proliferate, differentiate, or commit apoptosis. The 'self-optimisation' process underpinning these changes requires the continuous integration of spatial and temporal information from numerous sources and relaying this information to downstream effectors, via signalling networks [1,2]. Decades of biochemical studies have identified key players in these signalling networks and their interactions. However, most experiments have been performed using techniques that average over populations and/or are destructive. Such methods have largely alluded to, but not quantitatively described, the encoding of information in signalling dynamics, notably by applying external stimuli with varying temporal patterns [2–4]. However, without real-time measurements of signalling activity in single cells, the true dynamics of information flow within cells, and the heterogeneity of behaviour within populations, have remained largely concealed.

The advent of live single-cell imaging approaches now facilitates collecting data describing signalling network activity at sampling rates high enough to study the dynamics of information transfer in living cells [5–9]. This emerging field has seen rapid development in the number of measurable signalling elements [10], the length of time that dynamics can be analysed over [11], and the throughput of condition testing [12]. Analysis of these data is giving unprecedented insights into the fundamental systems whose emergent behaviour leads to robust fate changes [1,13,14]. With this understanding, 'designer' therapeutic interventions [13,15,16] and synthetic multicellular behaviours are now on the horizon [17]. Yet, major technical challenges still exist in converting live single-cell imaging data into biological conclusions.

In this review, we provide an overview of the current and emerging technologies that are now bringing us into an age where high-throughput live single-cell studies in human cells are possible. Additionally, we provide insights into the signalling fundamentals that these higher-throughput analyses are uncovering and how this knowledge is, in turn, driving further

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technological progress, thus bringing closer the possibility of large-scale data-driven models and maps of dynamic cellular signalling networks.

Expanding the Traceable Genome

Improvements in Protein-Tagging Systems

Central to live-cell imaging studies is the design of fluorescent reporters and 'tags' to measure signalling behaviour (Figure 1, Key Figure). Until recently, stable overexpression of fluorescent reporters represented the primary means of tracking protein levels in live single cells [18]. However, in these cell lines, the tagged protein is under the control of an artificial promoter, which could result in artificial dynamics. Proteins can now be efficiently tagged with fluorescent markers at their endogenous locus to determine their levels and localisation over time, under transcriptional control of their natural promoter (Figure 2A). Following pioneering studies (Box 1), the number of tagged proteins and reporters has expanded greatly [10]. In fact, in yeast, the relative ease of gene editing has allowed most of the proteome to be tagged [19]. This tagging has led to a dynamic analysis of the response of the yeast proteome to multiple stimuli, yielding a systematic inference of signalling protein function [20].

Technologies are emerging in metazoan cells that facilitate ever higher-throughput tagging of proteins, for example, through the generation of large libraries of tagged proteins [21,22]. In a notable study, random integration of tags, where a fluorescent coding region flanked by splice sites is integrated into the genome via a retrovirus, allowed nearly 1000 proteins to be tagged [23,24]. By automatically characterising protein behaviour following the application of DNA-damaging agents, Cohen *et al.* gained a systems-wide understanding of state changes in the DNA damage response. Moreover, the authors found the RNA helicase DDX5 to be a major antiapoptotic factor, demonstrating the strength of this approach in characterising in great detail the dynamic response of cells to drugs.

Box 1. Seminal Studies of Single-Cell Signalling Dynamics

Work exploring the dynamics of signalling within single cells has revealed fascinating insights into how cells regulate their fate and survive in ever-changing conditions. For example, early live-cell imaging studies facilitated modelling of gene regulatory networks in bacterial cells [89] and demonstrated how synthetic circuits could be designed [90]. Moreover, by using pairwise combinations of fluorescent reporters, Suel *et al*. revealed that different temporal dynamics of positive and negative feedback results in a system where cells stochastically transition into a temporally defined period of competence [91].

In mammalian cells, landmark studies showed both the NFκB pathway, important in stress and immunity, and the p53 pathway, involved in DNA-damage sensing, to have oscillatory behaviour [5,6,92]. Here, tagging both p53 and its inhibitor MDM2 in a single-cell line showed that these oscillations were due in part, to the participation of p53 in a negative feedback loop with MDM2 [5]. Meanwhile, tagging of NFκB and its inhibitor IκBa demonstrated that transcription of IκBa resulted in a negative feedback loop with NFκB. Together, these results demonstrate that oscillatory signalling dynamics may emerge in signalling pathways where negative feedback exists.

Finally, experiments looking at the kinase ERK found that both sustained and pulsatile activation were important in growth factor responses [93]. Moreover, cells modulate the frequency of ERK pulses to transmit information [94]. Albeck *et al.* also showed that downstream targets of ERK integrate the oscillating signal over time, suggesting that cells also decode information multiplexed in frequency-modulated ERK signals [94]. Live single-cell imaging studies have long been applied to the study of calcium signalling dynamics, using the fluorescent protein aequorin [95]. Interestingly, Hannanta-Anan and Chow recently used optogenetic control of calcium to show that the immune-related transcription factor NFAT integrates calcium signalling overtime, meaning this mechanism of temporal integration is likely not limited to ERK signalling [96].

These examples highlight how live single-cell studies have contributed greatly to our understanding of the complex signalling dynamics displayed in gene regulatory networks, and the mammalian NFκB, p53, and ERK pathways, as recently reviewed by Gaudet and Miller-Jensen [10].

Glossary

Cyclin-dependent kinase (CDK): a kinase that, when bound to cyclin, activates downstream transcription factors to mediate cell cycle progression.

Dynamic programming: an optimisation method to solve problems where many consecutive decisions need to be optimised over. Here, only the optimum path leading up to a decision is ever considered. For example, to find the optimum path between two huts over a narrow mountain pass, you do not need to consider all combinations of paths before and after the pass, only the best path to the pass from the first hut, and the best path from the pass to the second hut.

ERK: a kinase in the Ras-ERK pathway. This pathway converts information about extracellular conditions into transcriptional events. For example, extracellular growth factors bind to receptor kinases, which activate Ras and, ultimately, ERK; the activation of downstream transcription factors then promotes proliferation.

Förster resonance energy transfer (FRET): an imaging technique in which a donor molecule is excited with a high-energy photon, and the transfer of energy from this donor molecule excites a photon in the acceptor molecule. When this photon relaxes, light is emitted at a lower wavelength. Both the donor and the acceptor have to be in close proximity for this to occur.

Hidden Markov models: mathematical models of systems in which the state of the system is not exactly known, but exists with a certain probability, which depends on observations of the state and states before it.

K-nearest neighbour: an algorithm that searches for some number 'k' of the nearest neighbours to a given point; in cell tracking, this algorithm would search for the k nearest cells to the cell in the previous frame.

Kalman filter: a technique for determining the behaviour of unknown variables over time given a set of noisy measurements. Iteratively, the future values of the variables are predicted based on a model; these predictions are then updated at the next time point based on noisy measurements, and new variables are predicted. Importantly,

Major advances in genome engineering via CRISPR/Cas9 [25] are now expediting targeted endogenous gene tagging in metazoan cells. Recently, for example, Stewart-Ornstein *et al.* demonstrated the creation of a toolkit, termed 'eFlut', for tagging proteins using the CRISPR/Cas9 system with fluorescent reporters [26]. Leonetti *et al.* also developed an approach to higher-throughput tagging [27]. This study used a split GFP system, whereby GFP is separated into a small and large fragment, and proteins tagged with the small fragment subsequently fluoresce upon binding the large fragment [28]. The size of the small GFP fragment meant that Kamiyama *et al*. could use commercial CRISPR/Cas9 systems to integrate the small GFP fragment into genomic DNA [29]; this enabled them to tag 48 human proteins [27]. Importantly, eFlut and the split-GFP approach can use fluorescent proteins with different spectral characteristics, bringing large-scale multiplexed live-cell experiments closer.

To measure the activity of signalling enzymes, such as kinases and GTPases, specific reporters must be used. Systems based on **Förster resonance energy transfer** (FRET, see Glossary) are currently the furthest developed of these [30] (Figure 2B). Notably, following early work studying **Rho-GTPase** signalling in live single cells [31], spatiotemporal analysis of multiple FRET reporters, synchronised to protrusion formation, has offered fascinating insights into how Rho GTPases regulate the actin cytoskeleton [32,33]. Similar to protein tagging, FRET toolkits have been developed that allow systematic construction of reporters. For example, Fritz *et al.* developed a toolkit to generate and optimise FRET reporters of protein activity, demonstrated for reporters of **ERK** and RhoA activity [34].

However, limitations in both the signal:noise ratio and the number of channels that can be simultaneously quantified have driven the search for alternatives to FRET to measure signalling activity [35]. Specifically, efforts to measure cell cycle phase length and **cyclin-dependent kinase** (CDK) activity led to the development of reporters that translocate out of the nucleus upon phosphorylation [11,36,37]. Moreover, Regot *et al.* used this principle to develop kinase translocation reporters (KTRs; Figure 2C). Importantly, the authors harnessed the fact that KTRs use only a single imaging channel to simultaneously introduce and measure reporters for JNK, ERK, and p38 activity [35]. Such multiplexing can yield enormous amounts of information about how signalling pathways dynamically interact with one another, representing an important step forward in the field.

Capturing the Dynamics of Gene Regulatory Networks

Protein signalling networks often mediate long-term state changes by regulating transcription. To understand the dynamic crosstalk between signalling and transcriptional regulation, networks will be critical to gaining a systems-wide understanding of cellular information flow and how it effects changes in cell state.

RNA molecules lie at the heart of transcriptional regulatory networks. Technologies measuring RNA levels and localisation in live-cell studies are well known [38,39] and will likely be increasingly used in combination with reporters of signalling activity. The development of more scalable approaches to RNA tracking also represents a step towards such studies. Building from a system in which a specifically designed RNA aptamer, termed 'Spinach', fluoresces upon fluorophore binding, Paige *et al.* demonstrated endogenous labelling of single RNA molecules in living cells [40]. However, signal:noise limitations motivated the need to find alternative approaches [41]. Following demonstration that the endonuclease Cas9 can be targeted to specific mRNA molecules, which it subsequently cleaves [42], Nelles *et al.* developed an mRNA reporter by engineering a nuclear localisation signal onto Cas9-GFP with inactivated nuclease function [43]. When bound to the targeted mRNA, the mRNA and Cas9-GFP complex is exported from the nucleus, while unbound Cas9-GFP is reimported into the

the combination of the prediction and the measurement assigns a more accurate value to the variable than either could alone. In cell tracking, the location of a cell is an important unknown variable.

Nuclear factor kappa-light-chain enhancer of activated B cells (NFκB): this term refers to a family of proteins that regulates transcription, especially during immune and stress responses. Most single-cell studies have described the dynamics of a prototypical NFκB family member called RelA, or p65.

Optimisation: given a function or equation, numerical optimisation seeks to maximise the value output by the function by changing parameters. Global optimisation approaches deal with cases where there may be many local optima, analogous to finding the biggest mountain in a mountain range.

Rho-GTPase: proteins that are active when bound to GTP. Intrinsic catalysis of GTP to GDP then inactivates these proteins. Rho family GTPases are key regulators of cytoskeletal organisation, especially during cell morphogenesis and motility.

Segmentation: an image analysis term for marking out a specific region of an image, such as a certain colour or intensity.

Key Figure

A Live Single-Cell Imaging Workflow

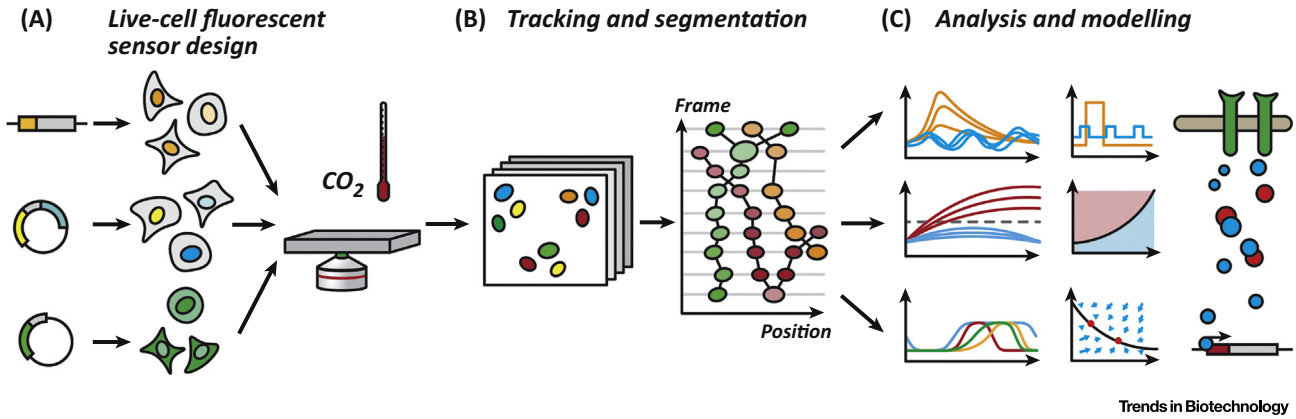

Figure 1. (A) Sensors, seeking to characterise behaviour of a specific signalling factor, are designed and introduced into cell lines. High-throughput live-cell imaging is then performed in atmospherically controlled conditions. (B) Image series are processed; subsequently, algorithms automatically segment cells, or their nuclei. Automated tracking algorithms then link segments to form tracks over the time course of imaging. (C) Features are extracted to quantify the fluorescent sensor over time. Mathematical modelling of the signalling dynamics and interpretation of the underlying biological mechanisms then guide further experimentation.

nucleus. Therefore, the nuclear:cytoplasmic Cas9-GFP ratio acts as a high signal:noise readout of mRNA level, which is demonstrated nicely for GAPDH mRNA [43].

Recent studies have also examined epigenetic regulation in live single cells. For example, Stelzer *et al.* introduced a fluorophore downstream of promoters known to be regulated by methylation. By showing that DNA methylation of a target site is carried over to the promoter for the fluorescent reporter, the authors developed a measure of DNA methylation [44]. This allowed the timing of a key demethylation event that occurs in the process of reprogramming somatic cells into induced pluripotent stem cells to be pinpointed, which had been previously masked by populational heterogeneity [44]. Also looking at epigenetic regulation, Bintu *et al*. developed a reporter for studying chromatin regulator dynamics in live cells [45]. Here, chromatin regulators are under tetracycline-inducible (TetR) targeting to the promoter of a fluorescent reporter. Upon chromatin regulators localising to the promoter, the reporter is silenced. Therefore, this method allows measurement of how quickly a chromatin regulator can silence a promoter. Combining protein tags and these reporters will allow us to study how signalling pathways impact longer-term changes to transcriptional regulatory networks.

Advances in Cell Tracking and Analysis

The drive for both higher-throughput live-cell studies and longer imaging time-series is leading to progress in the design and implementation of automated cell tracking algorithms, which are central to higher-throughput live single-cell imaging studies (Figure 1B). The fields of computer-aided cell tracking and **segmentation** are now well developed [46], and software for cell tracking has been compared for the first time [47]. However, major challenges remain in automating cell tracking and integrating tracking algorithms into data analysis pipelines [48].

Towards High-Fidelity Automated Cell Tracking

Generally, automated algorithms separate the cell tracking problem into two stages: (i) detection and segmentation of cells; and (ii) construction of tracks linking these detections over the

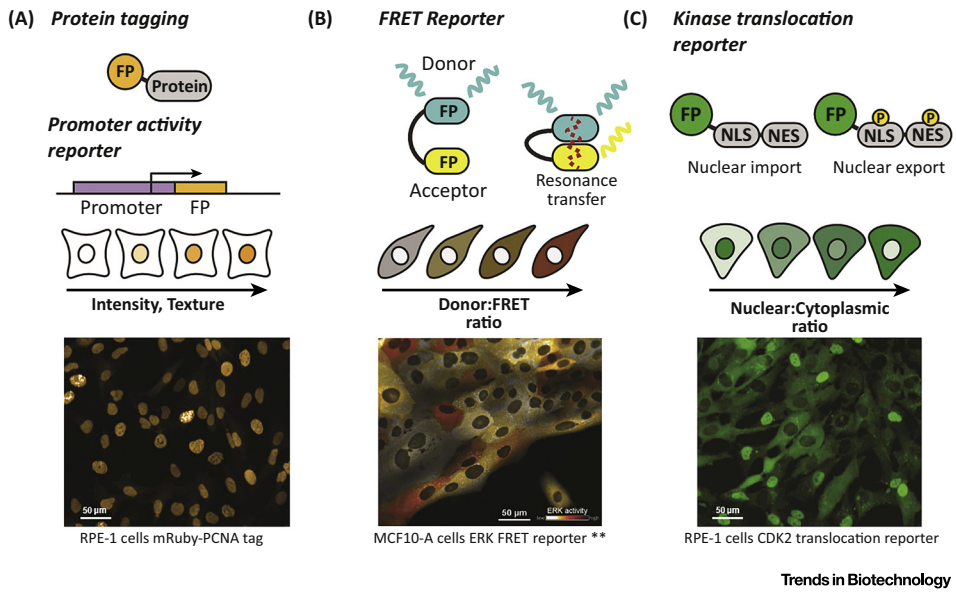

Figure 2. Fluorescent Sensors for Signalling Dynamics. (A) The levels and localisation of a protein may be determined by linking a fluorescent protein (FP) to the N or C terminus and measuring fluorescence intensity. To measure protein levels under the natural promoter, the fluorescent protein must be introduced at the endogenous locus. Intensity measurements can also quantify promoter activity directly by downstream insertion of a fluorescent protein. (B) Förster resonance energy transfer (FRET) reporters make use of resonance energy transfer between donor and acceptor proteins. When the donor protein is excited, if the acceptor is close enough, resonance transfer occurs and the acceptor emits at a lower wavelength. By engineering FRET reporters such that signalling activity alters the distance between donor and acceptor, the ratio of donor emission intensity to acceptor emission intensity can be used to determine activity. (C) Studies characterising the behaviour of proteins that translocate out of the nucleus upon phosphorylation have led to the development of kinase activity reporters (KTRs). Fluorescent protein is bound to an import-export protein domain. In the unphosphorylated form, the import signal is active and export signal masked; thus, import occurs. Following phosphorylation, the nuclear export signal dominates and export occurs. Kinase activity is then recorded as the cytoplasmic:nuclear (C:N) fluorescence ratio, where cytoplasmic intensity is quantified from a ring region around the nucleus. The dynamic range of a KTR describes the C:N ratio change between conditions of kinase activity and inactivity. Dynamic range improvements can be made by varying the phosphorylation site sequences [35]. Reproduced, with permission, from John Albeck (B). Abbreviations: NES, nuclear export signal; NLS, nuclear localisation signal.

period of imaging (Figure 3A). In the linking step, most algorithms either look for physical overlap between cell nuclei from one frame to the next or use **K-nearest neighbour** methods to minimise the movement and/or changes between segments in subsequent images, with rules handling events such as mitosis and death [46]. These algorithms are often successful in tracking separable cells imaged at high frequency. However, high cell densities and large movements frequently lead to detection errors. One way to combat this problem is to image more frequently, but high-frequency imaging limits throughput and can lead to phototoxicity problems [49].

To study live single cells in higher throughput and over longer time periods, there is a pressing need to develop algorithms capable of handling lower-frequency imaging rates and accounting for errors in cell segmentation [50]. Towards this end, **optimisation** approaches, now the gold standard in the field of multiple object tracking [51], are being applied to tracking in molecular biology. Such methods use a cost function to accurately quantify the quality of tracks over the time-series. Cost functions generally penalise large movements or gaps in tracks, while rewarding small movements and continuous tracks. An algorithm then seeks to globally optimise the cost function. Global optimisation approaches, as opposed to frame-to-frame linking, now dominate single particle tracking, as demonstrated in a recent objective

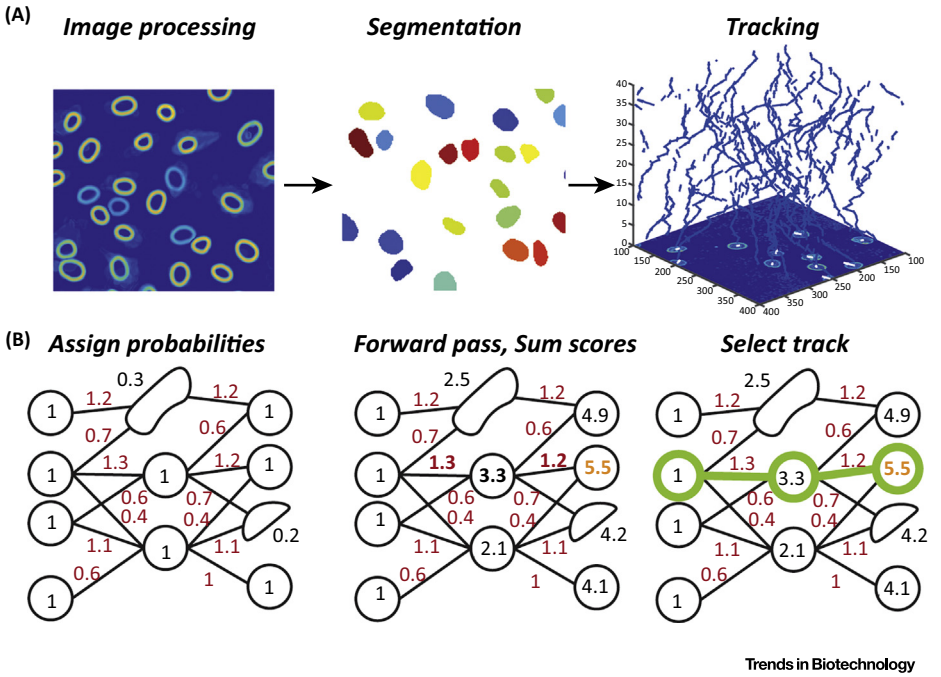

Figure 3. Automated Segmentation and Tracking. (A) In a standard workflow, initial image processing to highlight nuclei or cells is followed by automated segmentation of nuclei or cells; subsequently, algorithms are used to automatically track cells through the imaging sequence. (B) In a probabilistic tracking approach, each segment is assigned a probability of it being a correctly segmented nuclei, or circle in this example. Higher scores are also associated with shorter paths. A dynamic programming approach is then used to optimise the set of tracks. In the forward pass, scores are summed always taking the maximum value. In the reverse pass, the origin of the maximum score is traced back; this track is then included in the set of all tracks and the process iterated.

comparison of single particle tracking methods [52]. Here, multiple hypothesis testing and **Kalman filtering** approaches gave strong results, although no single optimisation method was best.

For cell tracking, Bise *et al.* [53] used a similar approach to that used by previous groups [54] to successfully track single particles. In these methods, track fragments are first generated, and then connections (including operations, such as merging, splitting, and mitosis) between these fragments are optimised via a **dynamic programming** approach (Figure 3B). Winter *et al*. used a similar optimisation approach for tracking neuronal progenitor cells [55], and Magnusson *et al*. developed a probabilistic, dynamic programming approach, which globally optimises tracks over the entire time course [56,57]. Notably, this software performed best in a recent benchmark test [47], demonstrating the power of global optimisation approaches to cell tracking.

Beyond dynamic programming approaches to optimising cell tracks, a rapidly developing trend in image analysis is the use of large neural networks, or deep learning strategies [58], recently applied to object tracking [59]. Such approaches have also been applied to the segmentation and detection of cells for high-throughput screens [60]. Thus, the field is now open to the application of such methods for live-cell tracking, where levels of quality that can currently only be reached by manual tracking may be achievable.

Cell Tracking in Pipelines

The accuracy of automated algorithms is currently below what is required for long-term tracking [48]; thus, there is still a need for solutions that use efficient manual correction. A recent study

[49] highlighted this problem and presented a tool for automatic segmentation, with subsequent manual tracking, or the option to import and amend tracks from other tools. Moreover, building on successful optimisation-based tracking, Winter *et al.* presented software that includes cell segmentation, automated tracking, and manual correction as an integrated package [61]. Beyond tracking itself, effectively identifying patterns in temporal data, and using these patterns to filter out tracking errors, can also improve accuracy. Towards this, Held *et al.* used **hidden Markov models** describing nuclear morphology dynamics over mitosis to select accurately tracked mitotic events [62]. Using this method, Schmitz *et al.* characterised how 675 different small interfering (si)RNA depletions affected mitotic progression [63]. In summary, integrating correction and improved filtering into tracking pipelines offers a ready solution to high-accuracy tracking where automated algorithms fall short.

Exploring Signalling Networks

Probing Signal Transmission

Transmitting information through signalling networks requires changing the activity levels of signalling elements, or nodes, in the network [12]. Many properties of signalling pathways can be characterised by using live-cell reporters to systematically quantify the response of signalling networks to varying inputs (Figure 1C). Importantly, live single-cell methods allow behaviours that would otherwise be masked in population-averaged experiments to be characterised, a key example being oscillations.

Microfluidic devices represent one of the major technologies facilitating experiments on signal transmission dynamics (Figure 4A) [64]. Such devices can quickly and accurately control external concentrations in chambers where live-cell imaging can be performed. In a key study of the yeast HOG pathway, which responds to osmolar shock, Hersen *et al.* developed a microfluidic device to rapidly change osmolarity [65]. By varying the frequency of osmolarity change, and by determining when the downstream transcriptional activity stopped matching input changes and instead began integrating the signal, the authors characterised the maximum rate (or bandwidth) that the pathway can operate at (Figure 4B). Hansen and O'shea also utilised microfluidics to study the bandwidth of the Msn2 pathway, involved in the yeast stress response [66,67]. Here, studying the behaviour of a large number of downstream targets of the Msn2 transcription factor demonstrated that the pathway can preferentially express four different types of gene depending on Msn2 translocation amplitude and dynamics, showing that cells may multiplex large amounts of information into a single pathway [68].

Following seminal work on the stress response factor **nuclear factor kappa-light-chain enhancer of activated B cells** (NFκB), showing it to have oscillatory dynamics (Box 1), Kellogg and Tay used a microfluidics approach to determine that NFκB oscillations display digital activation, where differing frequencies of activation induce early, intermediate, and late genes downstream [69,70]. Moreover, these oscillations can become entrained at specific frequency ratios with oscillating external stimuli, leading to enhancement of specific transcription factors [71]. While Zambrano *et al.* did not observe natural entrainment [72], both groups did observe three different transcription regimes, thus demonstrating that oscillations in transcription factor dynamics can create varying responses depending on input timing and frequency.

Optogenetics, where gene activity is engineered to be controlled by light-sensitive proteins, is another rapidly developing technology being used to study signalling pathway dynamics (Figure 4A) [73]. Following innovative early experiments looking at the dynamics of ERK activation that showed it to have oscillatory, all-or-nothing activation (Box 1), Toettcher *et al.* used optogenetic control of upstream Ras to study how varying the frequency of ERK activation affected transcription. By utilising a white noise signal (a combination of all

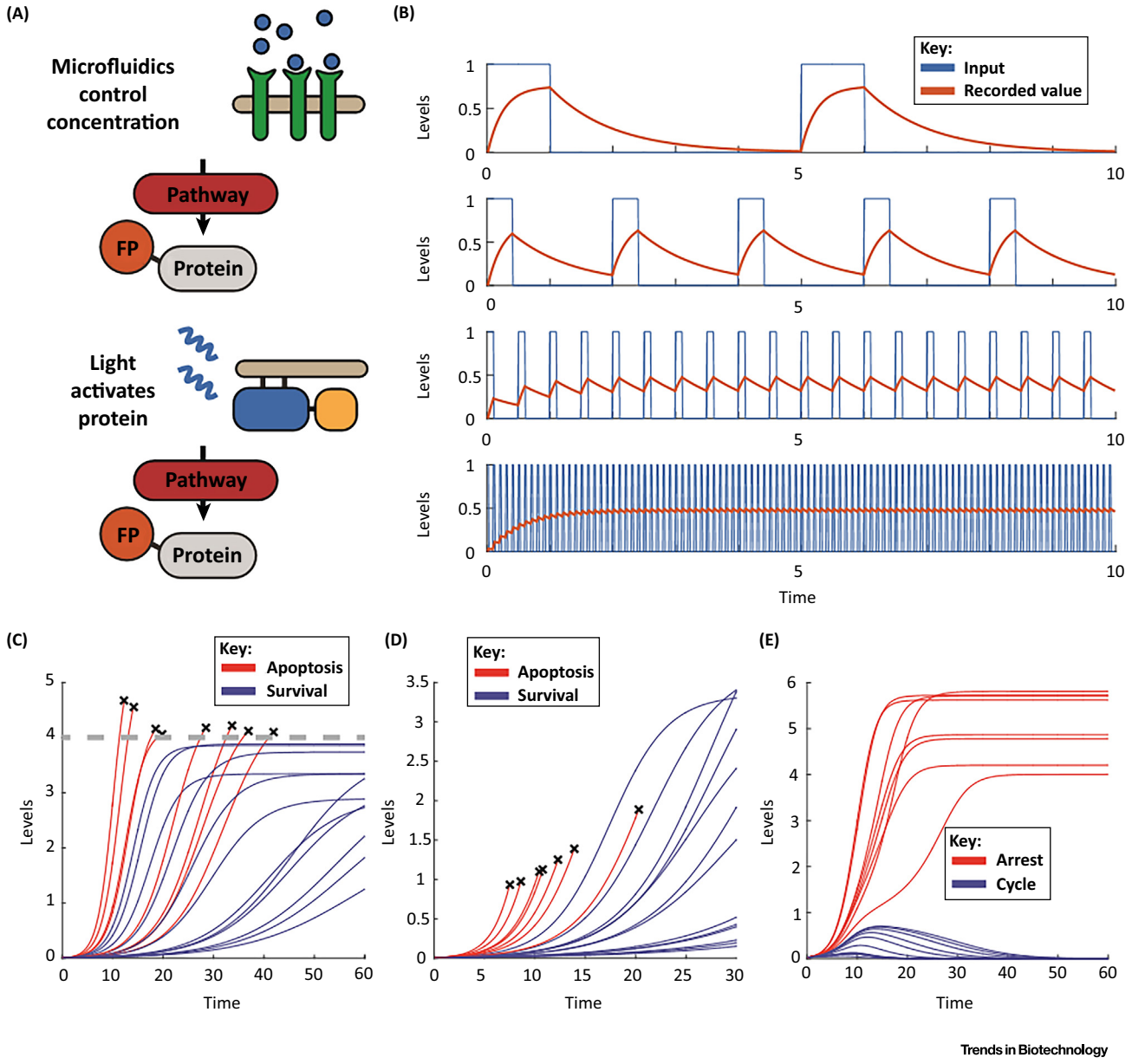

Figure 4. Analysing Signalling Dynamics. (A) Microfluidic devices that can accurately control extracellular signalling factor concentration, as well as proteins that can be activated optically, represent key tools for controlling input dynamics. By recording output dynamics at the single-cell level using a suitable fluorescent reporter (FP), an understanding of information flow in the pathway can be gained (Figure 2, main text). B) Quantifying the output of a pathway in response to an oscillating input (e.g., a square wave) can determine the rate at which a pathway can respond to input changes. This rate is intrinsically linked to the frequency of input at which the pathway stops responding to individual pulses, and instead begins to average and/or integrate the signal over time [65]. C) Live single-cell studies have shown that cells often make decisions based upon threshold levels of activity. In this example, apoptosis occurs when levels of a signalling factor exceed a threshold of 4 (a.u.) [78]. D) Live single-cell experiments have recently revealed that decisions can be determined by the rate at which protein and/or activity levels increase. In this graph, apoptosis occurs when the rate of increase exceeds a certain level [81]. E) Live single-cell studies have demonstrated that protein activity levels can 'bifurcate', where a population showing a single continuous distribution of activity separates into two distinct subpopulations. Identifying the point at which a cell is committed to one population or the other can allow the fate of a cell to be predicted long before systems-wide changes are observed. Here, for example, cells with high levels of a cell cycle progression inhibitor arrest, while those with low levels cycle [11].

frequencies) as the optical input stimulus, the authors established the fastest rate of signalling change that ERK can respond to. The authors also identified two groups of protein that responded to either transient or sustained Ras-ERK signalling, by monitoring Ras-ERK target phosphorylation levels, therefore demonstrating that variations in the dynamics of Ras-ERK signalling can lead to divergent responses. In an exciting new development, Hiratsuka *et al*. observed propagation of ERK pulses *in vivo* [74]. The authors found that, in normal epithelial tissue, ERK pulses stochastically arose in single cells and spread locally, while in wound healing, sustained ERK waves propagated through tissue. These results suggest that the divergent responses to ERK dynamics identified with optogenetics *in vitro* have a key role in modulating the effects of ERK dynamics *in vivo*.

Optogenetics have also been used to study the decision between commitment to stem or differentiated fates. Imayoshi *et al*. observed oscillatory behaviour in three genes (*Ascl1*, *Hes1*, and *Olig2*) that control neural progenitor differentiation into three respective lineages. Therefore, changes in the oscillatory regimen of one of these genes into sustained activity predict differentiation [75]. By optogenetically controlling Ascl1, the authors of this study showed that oscillations of Ascl1 are required for proliferation, and sustained activity induces differentiation, while unstimulated cells quiesce, meaning that cells can multiplex multiple fate outcomes into a single signalling pathway. Shimojo *et al.* engineered mice with progenitors that had optically controllable Dll1, another differentiation factor [76]. Here, the authors observed that sustained activity led to differentiation, while optical stimulation of oscillations maintained a proliferative state and severely impacted the neural development of the mice. As such, live single-cell imaging studies, coupled with technologies allowing us to map input spaces, are shedding new light on how information is transferred and integrated into decision making in a variety of cellular processes.

Predicting Cell Fate

A key goal in cell biology is to understand how changes to signalling input result in systems-wide effects that impact the fate of a cell, such as the decision to commit apoptosis. Live single-cell imaging studies are currently unique in relating the history of the signalling dynamics of a cell to its future fate (Figure 1C). Importantly, this paradigm allows researchers to study and modify the dynamic systems that underpin fate decisions.

For example, following previous studies of how cells undertake the decision to commit apoptosis in response to the TNF-related apoptosis-inducing ligand (TRAIL) [8,77,78], Roux *et al.* used a live single-cell FRET reporter for caspase protease activity to determine at what point caspase activity exceeds that required for apoptosis [79]. The ability to measure the dynamics of caspase 8 (C8) activity revealed that the maximum C8 activity reached, as opposed to total C8 activity over time, best predicted the apoptotic outcome (Figure 4C) [79]. The authors hypothesised that degradation of active C8 meant that its activity was not cumulative and, therefore, by inhibiting proteasomal degradation, C8 activity would become cumulative and TRAIL killing enhanced. The authors experimentally validated this and, thus, demonstrated synergy between TRAIL and inhibition of proteasomal degradation in killing cells.

Building upon pioneering work looking at p53 levels in live single cells (Box 1), Paek *et al.* found that the rate at which levels of p53 increase, rather than p53 levels themselves, best predicted apoptosis (Figure 4D) [80]. Furthermore, the authors hypothesised and verified that this effect was due to adaptive feedback control. In other words, p53 promotes the transcription of an apoptosis inhibitor, IAP, which then increases the future threshold level of p53 required for apoptosis [80]. In a related study of the p53-associated apoptotic decision, Chen *et al*. identified that, in single cells depleted of the p53 inhibitor MDMX, p53 undergoes an initial postmitotic pulse followed by low-amplitude oscillations [81]. Notably, the induction of DNA

damage in the postmitotic pulse led to cell killing, while cells in the oscillatory phase were resistant, which is perhaps the result of IAP upregulation [80]. Studying p53, Porter *et al.* used a live single-cell approach to show that p53 targets with a short mRNA half-life track p53 dynamics over time, whereas those with a long mRNA half-life act to integrate p53 activity over time [82]. Therefore, these studies provide mechanistic insight into how p53 targets, such as those modulating the apoptotic decision, respond differently to p53 dynamics.

Studying cell cycle regulation, Spencer *et al.* demonstrated, using a CDK2 reporter, that CDK2 activity levels shortly after mitosis predict whether a cell will proliferate or quiesce [11]. This prediction was made possible by the live-cell reporter revealing the formation of two stable subpopulations shortly after mitosis. One population had low CDK2 activity and entered quiescence, while the other displayed high CDK2 activity and continued to proliferate [11]. The formation of subpopulations requires the signalling system to have two stable states, a network property known as 'bistability' (Figure 4E). In a further study, Overton *et al.* modelled this system and found that double-negative feedback between CDK2 and the CDK inhibitor p21 underpinned this bistability [83]. Also studying cell cycle progression, Barr *et al*. integrated data from live-cell reporters of p27 and CDK2 to show that bistability emerges in the cell cycle between the G1 and S phase states due to double-negative feedback between the CDK inhibitor p27 and CDK2. Mathematical modelling showed that activity of the protein Emi1 ensures that transition from the G1 state to S phase state only normally occurs in the forward direction. Experimental depletion of Emi1 caused cycles of DNA re-replication, demonstrating the importance of Emi1 in maintaining the forward direction of the cell cycle [36]. In complementary work, Cappell *et al*. combined live single-cell imaging of G1–S progression with carefully timed perturbation experiments to show that Emi1 activity marks the point of irreversible S phase entry [84]. Overall, these studies show that, by studying the dynamics of signalling in single cells over time, we can use the history of the signalling of a cell to predict the future fate of the cell, and use this understanding to alter the fate of a cell, or even modify the underlying network to generate otherwise unseen behaviours.

Concluding Remarks and Future Directions

The experiments and work considered in this review highlight how live single-cell imaging studies are rapidly changing our view of cell signalling. No longer can we consider signalling as simple linear pathways of activation or inhibition. Instead, it is a complex, dynamic, nonlinear process of information integration and processing. In addition to taking steps to perform live single-cell studies *in vivo* [74,85], the field now appears to be moving from characterising individual motifs to understanding emergent properties of larger signalling networks, such as checkpoint control [86,87], or decisions to differentiate (see Outstanding Questions) [75,76]. In line with this development, major advancements in our understanding of signalling will come from integrating multiple reporters into single-cell lines, such as in pairwise combination with each other. Additionally, the ability to combine live single-cell imaging data with single-cell sequencing data will also lead to fascinating insights. For example, by combining novel techniques allowing genomic events to be identified in the history of a cell population [88] with live single-cell imaging data, we may be able to pin down the signalling changes that occur around the genesis of cancer. In conclusion, the accelerating rate at which live single-cell studies are being performed will no doubt lead to novel discoveries that will ultimately allow us to understand how the dynamics of signalling factors contribute to the behaviour of systems as a whole and how the dysregulation of signalling network dynamics underpins disease.

Outstanding Questions

Can we harness machine learning to fully automate the data extraction process, and even synthesise knowledge, novel hypotheses, and experiments?

Are signalling dynamics in single cultured cells predictive of single cell signalling *in vivo*?

Can we integrate live single-cell imaging data with other data modalities, such as sequencing and mass spectrometry, to improve our understanding of how changes to signalling dynamics affect cells and organisms at a systems-wide level?

What are the changes to signalling dynamics that occur during the onset of disease, and how can we use this information to improve therapeutic strategies in the clinic?

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