SYSTEMS BIOLOGY

Using movies to analyse gene circuit dynamics in single cells

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Abstract | Many bacterial systems rely on dynamic genetic circuits to control crucial biological processes. A major goal of systems biology is to understand these behaviours in terms of individual genes and their interactions. However, traditional techniques based on population averages 'wash out' crucial dynamics that are either unsynchronized between cells or are driven by fluctuations, or 'noise', in cellular components. Recently, the combination of time-lapse microscopy, quantitative image analysis and fluorescent protein reporters has enabled direct observation of multiple cellular components over time in individual cells. In conjunction with mathematical modelling, these techniques are now providing powerful insights into genetic circuit behaviour in diverse microbial systems.

As biologists, we must grapple with, and reconcile, two very different views of cellular behaviour. On the one hand, we frequently think of cellular functions as being determined by 'circuits' of interacting genes and proteins. In a loosely analogous way to electronic circuits, these chemical circuits encode genetic programmes that underlie differentiation, the cell cycle and other behaviours (FIG. 1a). They accurately respond to stimuli and generate precise behavioural programmes in individual cells. On the other hand, there is the 'noisy' view of the cell we get when we actually look at cells: they exist in squishy, dynamic and heterogeneous populations, the morphologies, gene-expression patterns and differentiated states of which differ from one another, even when environment and genotype are fixed (FIG. 1b). How can precisely defined genetic circuits give rise to heterogeneity and, conversely, how does heterogeneity affect the behaviour of biological circuits?

Movies offer a powerful way to address these questions (FIG. 1c). By engineering microbial strains to express fluorescent protein reporters for key genes, researchers can follow the changing characteristics of individual cells over time. Quantitative detection methods, improved microscope automation and software, and the range of fluorescent reporter genes that are now available, in conjunction with mathematical modelling, can be combined to analyse gene circuit dynamics. Together, these techniques allow researchers to characterize epigenetic states, identify new dynamic phenomena, analyse biochemical interactions within circuits and elucidate the physiological function of genetic circuits, all at the single-cell level. Finally, movies provide an aesthetically compelling view of cellular function that is often fascinating to watch. With movies, the eye often picks out subtle patterns in individual living cells that would be difficult to notice with less direct techniques. Few techniques are more fun.

How does quantitative movie analysis compare with alternative techniques for analysing gene circuits? Time-lapse microscopy follows a few genes over time in individual living cells. It complements approaches such as microarrays (which provide genome-scale expression data averaged over populations, but do not allow analysis of variability) and flow cytometry (which allows high-throughput acquisition of single-cell fluorescence values, but does not allow the same cell to be tracked over time). Movies also complement new single-cell quantitative PCR approaches, which enable analysis of expression of multiple genes in individual cells, but, because they require lysis of the cell, do not permit tracking of expression dynamics[1]. Movies enable researchers to determine the 'trajectories' of gene expression levels in individual living cells. One potential drawback of movies is that although many genes, and their expression levels, may be important for a particular process under study, most studies currently follow the dynamics of only a few genes at a time owing to the lack of distinguishable reporters. In the future, multispectral techniques may expand the number of simultaneous reporters[2]. However, studies that follow the dynamics of only two or three genes at a time can still be extremely informative.

Here we review work in which movies provide new insights into the dynamic behaviour of genetic components and circuits. For this Review, we have confined ourselves to microbial systems and have therefore

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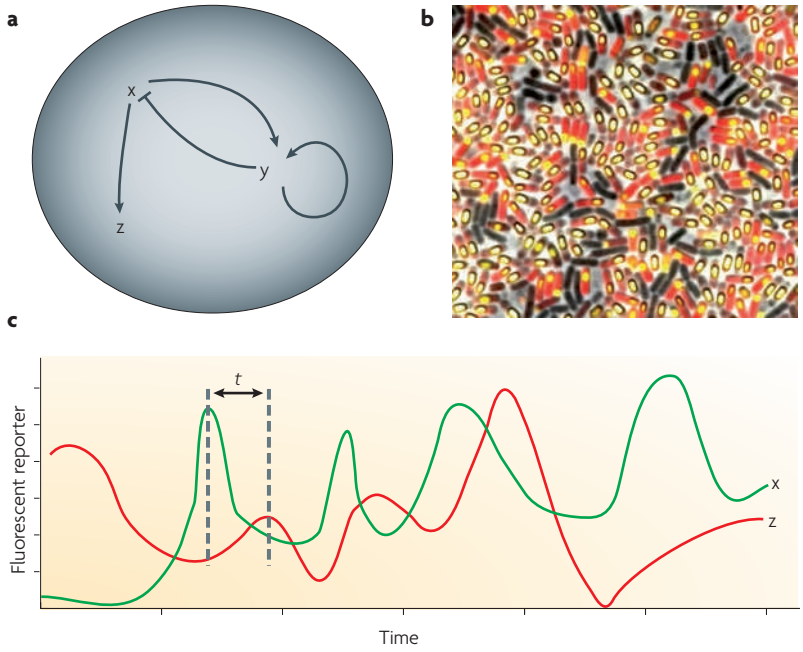

Figure 1 | **Circuit-driven versus noisy cells. a** | Circuit-level view: genes and gene products interact to generate an ordered behavioural programme. **b** | Noisy view: isogenic populations exhibit large levels of heterogeneity, both in terms of gene expression and differentiated states. As an example, we show an image of a *Bacillus subtilis* strain with two sporulation reporter constructs (P_{spoIIQ}–*cfp*, which is shown in yellow, and P_{spoIID}–*yfp*, which is shown in red), superimposed on a phase contrast image (shown in grey). Cells were grown in sporulation medium. However, they initiate sporulation at different times, which causes vegetative cells (dark rods) to coexist with cells at various stages of sporulation (coloured cells). **c** | Movies allow us to analyse the effects of interactions on the relative timing of gene expression in variable and dynamic circuits. Here, two schematic gene expression traces are shown in red and green for a simple activating interaction (x activates z). The movie enables us to observe delayed correlations that would not be evident in snapshots. *t* indicates a typical delay before the regulatory effects of x are visible in z.

excluded interesting recent work in mammalian cell culture and multicellular organisms[3–5]. Because our focus is on gene circuit dynamics, we have also excluded fascinating studies on the subcellular localization dynamics of individual cellular components[6–11]. We will first review recent work in which time-lapse microscopy has been used to examine and characterize variability in single-cell gene expression. Next, we describe how movies can facilitate quantitative analysis of biochemical interactions in individual cells. Finally, we explore how movies can provide integrated pictures of genetic circuit dynamics and thereby reveal key principles of genetic circuit design. Taken together, these studies are beginning to reveal intimate connections between the deterministic circuit and heterogeneous noisy views of the cell — although genetic circuits generate and control fluctuations in cellular components and heterogeneity in cellular states, variability is essential for at least some genetic circuits to function properly.

Noise
Fluctuations in molecular components. Noise arises owing to the low copy numbers of molecular species and the burst-like nature of transcription, among other mechanisms.

The origins of variability

If all cells behaved the same, it would be unnecessary to analyse individual cells. However, gene expression, and therefore cellular behaviour, is often variable, even in clonal cell populations grown in identical environments[12–14]. Where does this variability originate? It can arise from stochastic fluctuations, or noise, in cellular components and biochemical reactions[12,15]. These fluctuations are assumed to be significant given the low copy numbers of key molecular species in the cell[16,17]. However, variability can also reflect differences in the micro-environments inhabited by individual cells. Furthermore, pre-existing heterogeneity can be propagated to subsequent cell generations. Such effects can be observed by following lineages over several cell generations. The interplay between these sources of variability is addressed in several recent studies that used movies.

Lineage analysis allows tracking of epigenetic states. In the 1950s and 1960s, Powell[18,19] and others used phase-contrast microscopy to observe the growth of bacterial micro-colonies. They carefully analysed the heritability of growth rates and other phenotypes, and suggested that stochastic fluctuations in cellular components might generate the observed variability. Improved acquisition techniques (BOX 1; FIG. 2) now enable researchers to address these issues more systematically. Rather than tracking colonies for 2–3 generations by eye, it is now possible to record growth automatically over many generations[20].

Perhaps the best example of how lineage can affect cell–cell variability, which is observed in diverse multicellular systems and in yeast[21], comes from the study of ageing cells. For example, Ackermann *et al.*[22] took advantage of the asymmetric division cycle of *Caulobacter crescentus*, in which stalked cells divide to produce one stalked and one swarmer cell, to investigate the potential for ageing in bacteria. Stalked cells were followed by time-lapse microscopy for 300 hours as they divided repeatedly. Some of the swarmer cells they produced converted to new stalked cells that could also be tracked in the movie. The authors found that the cell-division cycle of stalked cells slows or stops over time, suggesting that stalked cells undergo senescence[22].

Although symmetrically dividing bacteria, such as *Escherichia coli*, seem to have no obvious means of ageing, cell-wall components may turn over slowly and be maintained in the poles where they are formed. By tracking over 35,000 *E. coli* cells using an automatic tracking programme, and using a flat micro-colony growth protocol (BOX 2), Stewart *et al.* showed that *E. coli* cells indeed age[23]. After division, each daughter cell has one new pole, created by the septation event, and one old pole, inherited from the parent. By sorting the lineage tree by pole age, one can observe that cells with older poles have systematically reduced growth rates, as well as increased death rates, compared with cells with younger poles (FIG. 3a). Without a specific molecular marker for cell age, it is difficult to imagine how such effects could have been observed using traditional techniques.

Persistence at the single-cell level. When antibiotics are added transiently to a population of bacterial cells, most of the population is killed. In some cases, however, a small percentage of 'persister' cells survive antibiotic treatments, grow and re-establish the population[24]. A second pulse of antibiotics leads to a similar result, showing that

Box 1 | **Movie acquisition and analysis techniques**

Movie acquisition

A minimal system for time-lapse microscopy consists of an automated fluorescence microscope, software to automate acquisition and software to assist in tracking and quantifying fluorescence in the resulting movies. The development of microscopes equipped with computer-controllable stages, filter wheels and shutters allows for the acquisition of movies of cell growth over several days. Diverse variants of GFP[86] now offer three or more distinguishable channels of information in the same cell[80]. The use of distinct localization signals can expand this repertoire[81]. Recently, software that automates time-lapse acquisition of images in various stage positions has become available both commercially and as a free open-source software package (μManager).

Movie analysis

Analysis of even the most breath-taking movies can require yawn- and repetitive-strain-injury-inducing amounts of time and mouse clicking. In many systems, the first stage of analysis is segmentation, which identifies the set of pixels belonging to each individual cell on each frame of each movie (FIG. 2). Segmentation has been performed successfully on both phase and fluorescent image data using various tactics, including: edge detection, thresholding and template-matching techniques, such as the Hough transform[87]. Once cells have been identified, tracking algorithms, such as those described in REF. 88, are necessary to infer cell lineages. Tracking can be a complex problem, as cells divide, grow, rotate, die and move irregularly. Most often, individual groups have developed in-house software to automate these processes for their particular imaging conditions[23,27,89]. However, it has been difficult to generalize these problem-, cell- and equipment-specific packages so that they can be used and adapted more broadly. Wang, West, You and colleagues have developed and distributed a package called CellTracer, which can be used with diverse organisms[90]. In addition, several other software packages are being developed and distributed to assist with this problem in yeast and mammalian cells[91,92], although these are not optimized for bacteria. Clearly, an organized community effort towards the common goal of developing a more general-purpose, user-friendly and open-source solution to this problem would be worthwhile.

persistence is not due to mutation[25]. But is persistence induced by the antibiotic, or do some cells spontaneously enter a persistent state before the addition of antibiotics? The persister state can be identified in movies because it causes strongly reduced cell growth. Using time-lapse microscopy of cells embedded in linear microfluidic chambers, Balaban *et al.* showed that individual *E. coli* cells switch in and out of the persister state spontaneously in the absence of antibiotics[26]. In a second study, the group used movies to identify an intermediate state in the transition to persistence during which cells stop growing but continue to express proteins[27]. Cells remained susceptible to antibiotics during this period.

This methodology could provide insight into biomedically relevant pathogens, including the slow-growing mycobacteria that are responsible for tuberculosis[24,28]. Being able to study rare spontaneous state changes could help identify strategies that influence the susceptibility of persistent infections to drug treatments. A current limitation of this approach is the difficulty in analysing extremely rare events, which become increasingly difficult to find at the low frequencies that characterize some natural persister states (for example, 10^{-6} for *E. coli*). To circumvent this problem, Balaban *et al.*[26] studied previously identified mutants that exhibit an elevated frequency of persisters[29]. With these imaging techniques established, an important challenge is to work out the underlying circuit that is responsible for inducing state changes in a probabilistic manner. Recent efforts have begun to identify new genes that can participate in such a circuit (reviewed in REF. 30). It will be interesting to see whether this circuitry is similar to the excitable genetic circuit that is responsible for the similarly transient and probabilistic process of competence induction in *Bacillus subtilis* (described below).

Heritability of cellular states. Cell-state heterogeneity can be analysed without movies, but movies can provide additional insights into the process. For example, Acar *et al.* recently analysed the galactose utilization system in yeast[31]. They showed that yeast cells with mutations in one feedback loop appear to spontaneously switch between states of high and low expression of galactose utilization genes. But it remained unclear whether (and how) these states are inherited across cell generations (or division events). Kaufman *et al.* addressed this issue by examining the heritability of such gene expression states[32] using movies to track yeast cells over 15 hours through ~6 divisions (FIG. 3b–d). Remarkably, mother and daughter cells switch on the galactose pathway synchronously after division, indicating that the timing of these apparently random decisions is heritable. The authors explain this behaviour in terms of a model based on a single fluctuating regulatory protein that is synthesized in large bursts. Clearly, the interplay between stochastic switches and heritable states can be complex.

Cell cycle variability. As an unsynchronized, dynamic oscillatory process that is continuously operating in individual cells, the cell cycle represents a key potential source of variability. But how variable is the cell cycle itself? And where does that variability originate? When examined at the single-cell level, cell cycle progression can be strikingly variable. Movies have been used to quantify variability in the timing of specific cell cycle stages in yeast[33,34]. This work broke overall variability in timing into steps that were either dependent or independent of cell size. The first step is responsible for controlling cell size before division. Interestingly, variability in the cell-size-independent time interval was reduced by increasing ploidy in a manner that was consistent with stochastic variation in expression of certain genes, such as those that encode G1 cyclins.

In a complementary study, Ramanathan and co-workers dissected the timing variability that leads to the cell-fate decision in yeast to initiate meiotic sporulation[35]. This study showed that the timing of sporulation varies considerably among cells, even though all cells activate expression of the master regulator of sporulation at roughly the same time. Variability in the decision to initiate sporulation results in part from slow and variable accumulation of the master regulator Ime1p.

These studies, together with another study[36], have begun to examine molecular mechanisms by which cell cycle progression can be intrinsically noisy. It will be interesting to see how this variability affects other cellular processes and to what extent this variability is adaptive.

Movies of clocks. In contrast to the inherently variable oscillations of the cell cycle, circadian clocks possess remarkable temporal precision. Cyanobacteria possess a 24-hour circadian clock, the molecular components of which

Segmentation
Breaking up a complex image into individual objects, such as cells.

Edge detection
A computational algorithm that identifies sharp changes in intensity associated with boundaries between objects, such as cells.

Thresholding
One of the simplest segmentation techniques, in which groups of pixels for which the intensity exceeds a defined cut-off value are identified.

Hough transform
An algorithm for identifying particular shapes, such as circular disks, in complex images. The Hough transform is useful in many segmentation systems.

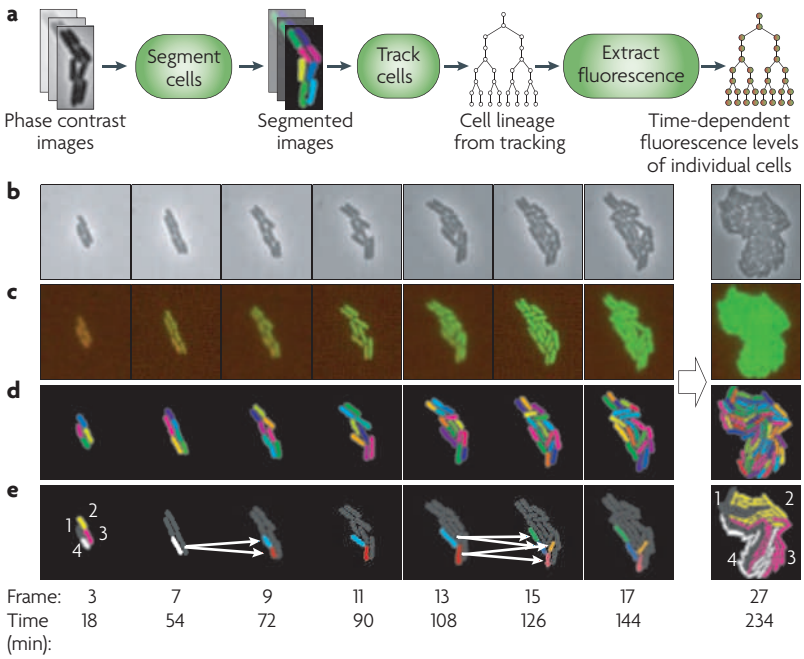

Figure 2 | **Tracking and segmenting single cells. a** | Schematic of data flow for a cell tracking and segmentation system. During tracking, cell shapes must first be identified in images (segmentation) and then tracked over time. Finally, the fluorescence values must be extracted. **b**–**e** | Segmentation and tracking input and output. **b** | Phase contrast images over time. **c** | Fluorescence images of the micro-colony. In this example, filters for yellow and cyan fluorescent proteins were used (shown in red and green, respectively). **d** | Segmentation was performed on the phase contrast images to determine the locations of each cell. Arbitrary colours were used for labelling. **e** | Shows the descendents of cell 4. The panel on the far right shows the descendants of each of the four initial cells after approximately four generations. Figure is courtesy of J. Young, California Institute of Technology, California, USA, and N. Rosenfeld, Rosetta Genomics, Rehovot, Israel.

Linear microfluidic chamber
A microfluidic device in which cells are confined to grow in a narrow groove. These devices facilitate analysis of cell lineages, as more closely related cells are located closer together.

Galactose utilization system
A system of genes used by yeast to control the uptake and metabolism of galactose. This system is characterized by several feedback loops, both positive and negative.

Repressilator
A synthetic genetic circuit designed to produce clock-like oscillations in the levels of its components. The circuit consists of a 'rock–scissors–paper' feedback loop of three repressors, in which the first represses the expression of the second, the second represses the expression of the third and the third represses the expression of the first.

have been identified and shown to reconstitute oscillations *in vitro*[37,38]. Although colonies and cultures exhibit robust rhythms, it was unclear how accurately the clock performed at the single-cell level, given the possibility of substantial noise in the levels of its molecular components[39–41].The possibility that the circadian clock might be inherently noisy was also suggested by studies of an unrelated synthetic genetic clock, termed the repressilator. In movies, the repressilator spontaneously generates self-sustaining (limit cycle) oscillations in *E. coli* cells without external perturbations (that is, oscillations do not 'damp out')[42]. However, the clock is erratic and its state quickly becomes desynchronized between cells within the population.

This work raised some questions. What are the limits of accuracy for simple synthetic clock circuits in individual cells? Do natural biological clocks function more accurately than synthetic clocks? And, if so, how? New work from Stricker and colleagues addresses the first question[43]. The authors analysed a synthetic clock design that was based on the activator AraC and the repressor LacI. AraC activates its own expression as well as that of LacI. Similarly, LacI represses itself and the activator. Resembling a 'relaxation' oscillator, and as predicted computationally, this design produced more-accurate oscillations in individual cells than did the repressilator[44,45]. Also, the period of these oscillations could be tuned using arabinose and isopropyl-β-D-thiogalactopyranoside. Another feature of this work was that the authors acquired movies of these circuits using a special microfluidic device that permitted chemostatic conditions but maintained flat cell growth, which enabled quantitation of fluorescence.

In contrast to the variability that is generally observed with synthetic clocks, when Mihalcescu *et al.* used a sensitive luciferase reporter system to image cyanobacterial clock dynamics in individual cells of growing micro-colonies over time[46], they found that all individual cells oscillated with a robust 24-hour rhythm. Furthermore, when two micro-colonies previously entrained to different clock phases were placed next to each other on the same pad, they did not influence each other's phase. These results suggested that the clock behaves accurately in each individual cell and does not require intercellular communication for synchronization[46,47]. This accuracy occurs despite noise in the gene expression from clock-controlled promoters, which was also recently analysed using movies[48].

How, then, does the cyanobacterial clock function so reliably? Recently, Rust *et al.* established a clock mechanism based on phosphorylation interactions among the three key proteins KaiA, KaiB and KaiC[38,49]. The post-translational nature of this clock circuit mechanism may help to explain its apparent robustness to noise. Ironically, the high intrinsic accuracy of the clock, which has been established using movies, may enable researchers to study many aspects of this system without movies — that is, at the population average level.

Together these studies represent a range of examples in which variability is generated or suppressed in different circuits. They therefore raise the question of how such circuit-level variability emerges from specific biochemical interactions in the cell.

In vivo biochemistry

Surprisingly, movies can provide the type of detailed and quantitative analyses of biochemical interactions that are traditionally associated with gels, blots and other biochemical assays. Several recent studies push the limit on quantitative analysis of movie data.

The gene regulation function. One of the most basic characteristics of a genetic circuit is the interaction between a transcription factor and its target genes. This interaction can be summarized by the effective gene regulation function (GRF): the relationship between the concentration of one or more transcription factor in a cell and the rate of production of its target gene. The shape of the GRF is crucial for the function of gene circuits. However, population-average measurements can 'smear out' even a well-defined continuous response function, and *in vitro* measurements may not reflect the many effects of the intracellular environment. These considerations motivated an effort to measure GRFs in individual living cells.

To measure a typical GRF, Rosenfeld *et al.*[89] engineered a strain of *E. coli* in which a lambda repressor–yellow fluorescent protein (YFP) fusion protein repressed the expression of cyan fluorescent protein (CFP), which

Box 2 | **Issues that impact time-lapse analysis of individual cells**

Reporter type
To directly monitor protein levels, fluorescent protein genes can be fused to proteins of interest (protein fusion). Alternatively, such genes can be inserted downstream of an additional copy of a natural promoter (promoter fusion), which is less likely to disturb the function of the cell but does not permit analysis of post-translational dynamics, including localization and degradation.

Protein stability
If promoter fusions express stable reporter proteins (GFP and its derivatives are generally stable in bacteria), then it may be difficult to visualize a rapid turn-off in expression owing to lingering previously expressed proteins. In such cases, one can analyse the rate of protein accumulation (time derivative of fluorescence), rather than the amount of protein[93]. On the one hand, protein stability can be an advantage, as it removes uncertainty in the degradation rate. On the other hand, time derivatives amplify measurement noise in image data. Another strategy is to destabilize the reporter protein using genetic tags, such as those derived from *ssrA* in bacteria[94]. This increases time resolution at the expense of reduced fluorescence levels.

Maturation time
Fluorescent proteins require widely varying times (minutes to hours) to become fluorescent, and therefore fluorescence must be directly measured in the strain of interest. Slow maturation can 'smear out' otherwise rapid events in individual cells.

Other issues
Fluorescent reporter genes may be subject to unknown interactions with endogenous cellular components, and in some cases, may depend on ionic conditions[95]. Controls, for example with constitutive or inducible promoters, should therefore be performed to make sure that the reporter proteins are behaving as expected.

Growth considerations
Growth of cells on a two-dimensional surface permits use of simple, rapid and light-efficient wide-field fluorescence microscopy. However, cells eventually 'pile up', limiting the length of time of observation. Microfluidic chambers have been introduced to maintain chemostatic conditions[26,83]. In our work, we find that imaging cells between the coverslip and an agarose pad of a few millimetres provides a relatively simple means of sustaining two-dimensional growth for many cell generations. Although this system is imperfect, it is sufficient for many applications.

was expressed from a lambda-regulated promoter. They allowed the tagged repressor to dilute out as the cells grew into micro-colonies, while simultaneously monitoring the rate of increase of CFP fluorescence (FIG. 4a,b; Supplementary information S1 (movie)). These data give the GRF for the lambda repressor–promoter interaction. Crucially, they showed that the GRF was not a well-defined function. Rather, CFP expression fluctuates slowly, with a typical timescale of one cell cycle. Thus, the output of the repressor–promoter system is not solely determined by the concentration of repressor in the cell. Because these fluctuations are slow, the cell would require several generations to 'average them out'. In this way, GRF fluctuations fundamentally limit the accuracy of transcriptional regulation. Single-cell movies therefore permit a more complete understanding of both the mean GRF, which is useful for approximately analysing gene circuit models, and the characteristics of its fluctuations, which are necessary for modelling stochastic effects.

The measured GRF had a remarkably accurate predictive value: when synthetic negative-feedback circuits were constructed using only the measured promoter, they exhibited the mean expression levels and noise expected from a simple genetic circuit model, with no free parameters[50]. In related work, Austin *et al.* used movies to measure the timescale of noise in simple synthetic gene circuits in growing *E. coli*[51]. They showed that negative autoregulation shifts noise to higher frequencies, which are easily filtered out by genetic networks. This represents an additional functional role for negative autoregulation, a common motif in natural circuits. This integrated analysis of regulatory components and noise will probably facilitate quantitative synthetic biology approaches[52–54].

A similar example in which movies were used to characterize biochemical systems that are useful for synthetic biology was recently undertaken by Grilly and co-workers[55], who characterized a prokaryotic protein degradation system in yeast. To determine the protein degradation rates, they simply tracked the fluorescence of a GFP construct tagged for degradation in single cells[55].

Single molecules: the final biochemical frontier. Perhaps the ultimate limit to *in vivo* analysis of biochemical interactions is being able to resolve circuit dynamics at the level of individual molecules in a cell. This milestone was recently reached in several pioneering studies of *E. coli*, all of which used movies to quantitatively analyse the production and degradation of individual mRNA or protein molecules[56–58]. First, Golding *et al.*[56] examined the production of mRNAs over time using a fluorescent protein fusion to the sequence-specific RNA-binding protein MS2. Cells were engineered to co-express target mRNAs that contained an array of MS2 binding sites. When present, these target mRNA molecules concentrated the fluorescent MS2 protein into 'dots' that could be imaged and counted inside cells over time. At the protein level, one might simply try to detect individual fluorescent protein fusions. However, if they diffuse freely in the cytoplasm, the signal they produce before photobleaching is effectively dispersed over the area of the whole cell. As a result, the fluorescence per camera pixel is well below the background autofluorescence of the cell itself, and an individual fluorescent protein molecule is practically undetectable. To circumvent this problem, Yu *et al.*[57] fused YFP to a membrane protein to reduce its mobility in the cell, which caused the time-averaged fluorescence from each molecule to be detectable[57] (FIG. 4c). This strategy can be generalized, as various different proteins can be fused to immobilizing domains. In a complementary study, Cai *et al.*[58] also measured the activity of individual molecules of the well-characterized enzyme β-galactosidase by monitoring the rate of enzymatic cleavage of a substrate to a fluorescent product. Because cells were confined to microfluidic chambers, the rate of accumulation of fluorescence could be monitored in movies for individual cells[58].

Together, these studies showed that researchers can track the production and degradation of individual mRNA and protein molecules over time using movies. In addition, the results obtained with these methods have enabled the development of predictive models for gene expression. These experiments show that both mRNA production and translation occur in exponentially distributed bursts[59]. The variability in expression caused by both types of bursts can be approximated by a gamma distribution, which is characterized by two effective parameters: the frequency of transcriptional bursts and the mean number of proteins produced per burst[56,59,60].

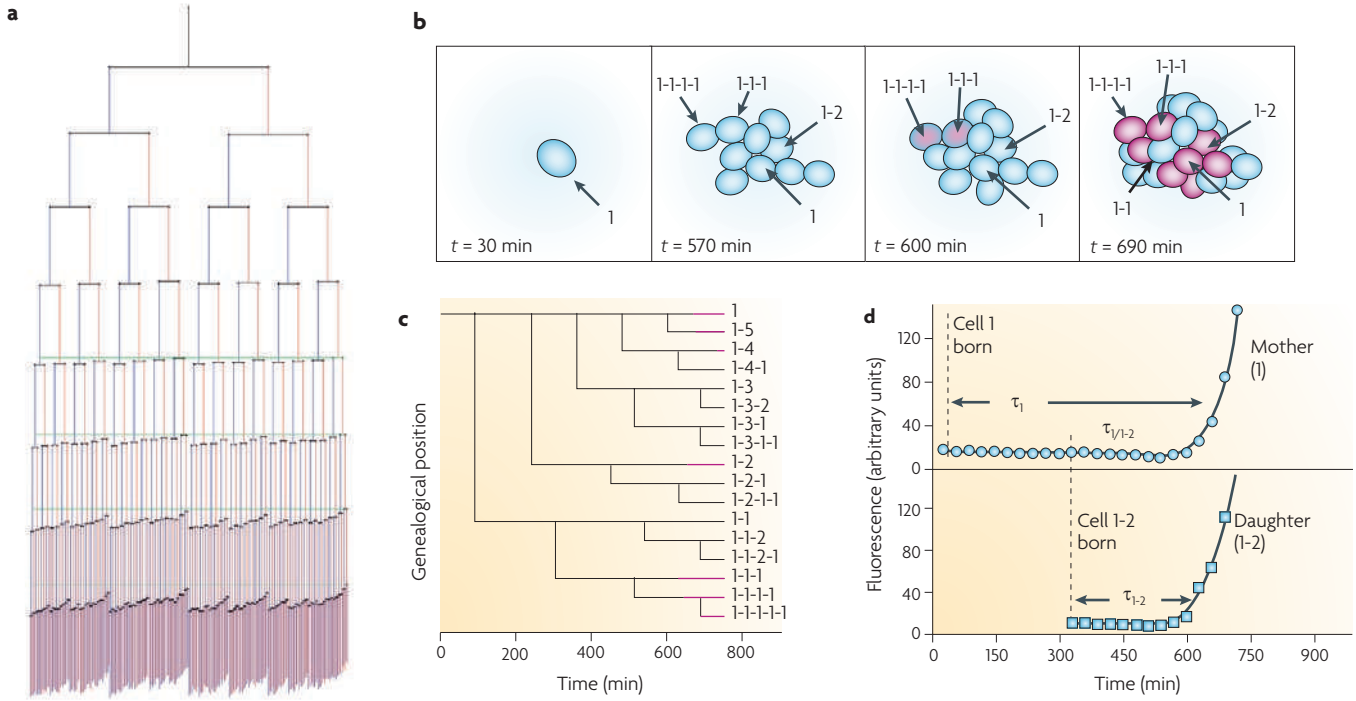

Figure 3 | **Automated lineage analysis reveals epigenetic states. a** | The ageing of *Escherichia coli*. This lineage tree depicts 9 generations of *E. coli* from 94 movies. The lengths of the lines joining cells to their progeny are proportional to the average growth rate of that cell, so a shorter line represents a slower growth rate. At each division event, the cell that inherits the old pole is shown in red and placed on the right side of the division pair, whereas new poles are shown in blue and placed on the left side of the division pair. Horizontal green lines mark the point of the first cell division for the last four generations. This lineage tree clearly shows that cells with older poles have systematically reduced growth rates compared with cells with younger poles. **b–d** | Genealogical switching history in the yeast galactose system. In each movie, the first cell is designated cell number 1 and sequential daughters of that cell are designated 1-1, 1-2, 1-3 and so on. These daughter cells bud in turn, giving rise to cells 1-1-1, 1-1-2, 1-2-1 and so on. **b** | Yeast cells that express a reporter for the galactose (GAL) system, P_{GAL1} – yellow fluorescent protein (YFP), in a mutant background in which a negative-feedback loop has been disrupted. Purple shading represents YFP expression. An initially 'off' (non-expressing) cell grows into a variegated micro-colony. Beginning at 600 min of age, or after 4 generations, several cells fluoresce almost simultaneously. This includes the mother–daughter pairs (1,1-2 and 1-1-1,1-1-1-1). **c** | The family tree for the colony shown in part **b**. Off cells are marked with a black line, whereas pink lines represent cells that express the GAL system. **d** | Fluorescent time courses for mother cell 1 and her daughter 1-2, showing each as they switch into the 'on' state. Mother and daughter cells switch on the GAL pathway synchronously after division, indicating that the timing of these apparently random decisions is heritable. Panel **a** is reproduced from REF. 23. Panels **b–d** are reproduced from REF. 32.

Together, these studies, and others like them[61,62], will help bridge the gap between the biochemical interactions between individual molecules, as they occur in cells, and the behaviour of more complex circuits that consist of multiple components and interactions.

Movies reveal circuit dynamics

Various techniques have begun to reveal the structure of genetic circuits: what regulates what, and how. However, despite this information it remains difficult to predict the cellular dynamics that a particular circuit will generate. This is especially true for circuits that are only active in a subpopulation of cells and for circuits that have highly variable behaviours. In *B. subtilis* under stress, a small percentage of cells (5–10%) enter a state of competence, during which they can readily take up exogenous DNA[63,64]. Similarly, *B. subtilis* undergoes a dramatic differentiation process in which a cell transforms into a dormant spore. Individual cells vary significantly in when, and in some cases whether, they initiate sporulation. What accounts for this variability in cell-fate decision making? Recently, movies have enabled researchers to connect the decision-making behaviours of individual cells to the architecture of underlying genetic circuits in both competence[65,66] and sporulation[67].

Transient, probabilistic differentiation. Pioneering work by Dubnau, Zuber, Grossman and others established the key molecular interactions that are necessary for competence (reviewed in REF. 68). The transcription factor ComK is both necessary and sufficient to induce *B. subtilis* cells into the competent state, and positively autoregulates its own expression. Expression of ComS is necessary, but not sufficient, for inducing competence. In movies, cells appear to spontaneously activate ComK and, after some time, revert back to vegetative growth. What accounts for the probabilistic, unsynchronized activation of competence in only a fraction of cells? And, how do cells ensure a timely exit from the competent state?

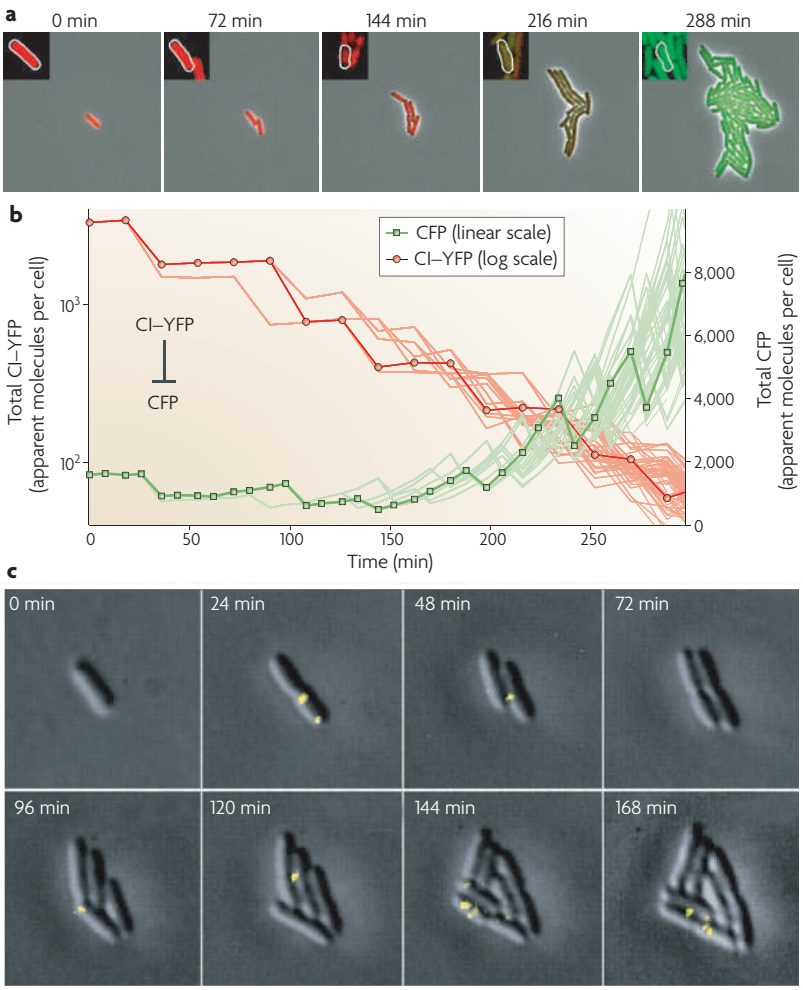

Figure 4 | ***In vivo* biochemistry. a**,**b** | Measuring the gene regulation function (GRF) of a repressor–promoter interaction in individual *Escherichia coli* cell lineages. Here, CI–YFP (lambda repressor fused to yellow fluorescent protein) represses expression of cyan fluorescent protein (CFP). In the regulator dilution experiment, cells are transiently induced to express CI–YFP and are then observed using time-lapse microscopy as this repressor dilutes out during cell growth. Part **a** shows a filmstrip of a typical experiment. CI–YFP is shown in red and CFP is shown in green. Part **b** shows quantitation of the movie. CI–YFP levels decrease by dilution (red lines), eventually permitting expression of the *cfp* target gene (green lines). The darker lines correspond to the cell lineage shown in the insets to part **a**. **c** | Monitoring transcriptional bursts in single cells. Frames from film footage of the expression of Tsr–Venus under the control of a repressed *lac* promoter. Tsr–Venus expression is shown in yellow and is overlaid with simultaneous DIC (differential interference contrast) images (grey). Note the burst-like expression pattern. Parts **a**,**b** are modified, with permission, from REF. 89 © (2005) American Association for the Advancement of Science. Part **c** is reproduced, with permission, from REF. 57 © (2006) American Association for the Advancement of Science.

Movies of *B. subtilis* cells containing reporters for pairs of genes during entry and exit from competence provided clues to address these questions. For example, cells exhibited a strong anti-correlation between ComK and ComS: as soon as ComK levels switched 'on', ComS expression switched 'off', and vice versa (FIG. 5a–c; Supplementary information S2 (movie)). This is consistent with the idea that ComK directly or indirectly represses the expression of ComS to form a negative-feedback loop. When combined with positive autoregulation of ComK, the two feedback loops together explain the probabilistic and transient nature of competence in terms of excitable dynamics that are mathematically similar to action potentials in neurons[69]. But what is the 'trigger' for differentiation? In the mathematical model of competence that was based on excitability, noise — that is, fluctuations in molecular components, such as ComK — triggers differentiation. To test this idea, cells that were mutant for the septation gene *ftsW* were used. These cells displayed normal physiology, but lacked the ability to septate, and therefore grew into long filaments. Because of their increased size, they exhibited reduced noise (fluctuations in gene expression averaged out more in the larger cells) but had similar mean levels of gene expression. They also exhibited a progressively reduced propensity to differentiate at longer lengths, supporting the idea that fluctuations are necessary for differentiation in this system[66].

Dubnau and colleagues[70] approached the question of whether noise is required for differentiation from a complementary and more direct point of view: they tested the hypothesis that fluctuations in ComK expression were responsible for initiating differentiation. They generated strains that differed in the noisiness, but not the mean rate, of ComK expression. By reducing the rate of ComK translation while increasing its rate of transcription, the expression of ComK was made less 'bursty'. Strikingly, the frequency of differentiation was reduced[70]. These experiments established that ComK noise affects differentiation propensity. This approach could be extended to test the role of noise in other factors, as well as in competence and in other systems[64,70].

Sporulation: terminal differentiation. In *B. subtilis*, entry into the sporulation pathway is controlled by the master transcription regulator Spo0A, the expression of which is heterogeneous[71,72]. Veening *et al.*[67] used time-lapse movies of a strain with a fluorescent *spo0A* reporter gene to analyse the decision of individual cells to sporulate (or not sporulate) (FIG. 5d). Their movies revealed that *B. subtilis* implements a 'bet hedging' strategy, whereby some cells sporulate (high Spo0A activity), while others use alternative metabolites to continue growing (low Spo0A activity). By analysing cell lineages, they showed that variations in the propensity to sporulate persist for up to two generations. Interestingly, these results are similar to those seen for the inheritance of states in a bistable switch in yeast[32].

Spo0A becomes transcriptionally active upon phosphorylation by a multi-component phosphorelay[73]. Multiple feedback loops influence Spo0A activity, including direct autoregulation of Spo0A. By replacing the P_{spo0A} promoter with a constitutive inducible promoter, Veening *et al.* showed that the autoregulation of Spo0A is not responsible for bistability of Spo0A activity or for epigenetic inheritance[67]. In addition, when they replaced Spo0A with a mutant that phenocopies the phosphorylated form, effectively removing the influence of the phosphorelay, they found that all cells increase expression of Spo0A similarly. Thus, movies enabled the authors to determine which circuit interactions are required for heterogeneous differentiation.

Spo0A
The master transcriptional regulator for sporulation in *Bacillus subtilis*. Spo0A is controlled by phosphorylation and transcriptional regulation.

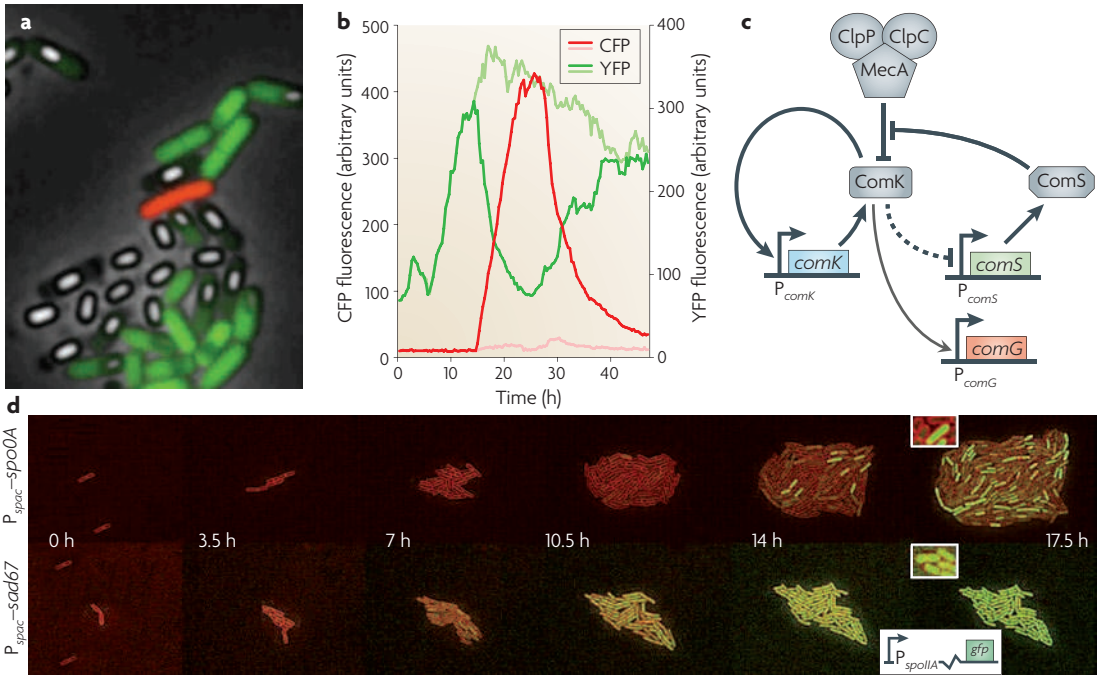

Figure 5 | **Circuit-level dynamics. a–c** | Analysis of *Bacillus subtilis* competence circuit dynamics in individual cells. Part **a** shows a snapshot from a movie. P_{comS} expression is shown in green and P_{comG} expression is shown in red. The red cell is in the competent state (high ComK levels). White depicts spores or sporulating cells. Part **b** shows a quantitative time series of P_{comS}–yellow fluorescent protein (*yfp*) (green lines) and P_{comG}–cyan fluorescent protein (*cfp*) (red lines) for the competence event shown in part **a**. Note the anti-correlation in expression between the two promoters, which can be explained by the circuit diagram in part **c**. P_{comS} and P_{comG} activities obtained from the non-competent sister cell are shown in light green and light red, respectively. Part **c** shows a map of the effective regulatory interactions in the core competence circuit in *B. subtilis*. The dashed inhibitory arrow depicts indirect repression. ComS competes with ComK for degradation by the MecA–ClpP–ClpC complex, effectively stabilizing ComK. **d** | The *B. subtilis* phosphorelay is required to generate variability in sporulation in *B. subtilis*. Time-lapse microscopy shows that heterogeneity in this system does not require the positive-feedback loop of Spo0A on itself (top row), but does require the activity of the phosphorelay (bottom row). Membranes are stained with FM5–95 (red), and expression of the sporulation reporter P_{spoIIA} is shown in green. The insets show a close-up of the cells. Panels **a–c** are modified, with permission, from *Nature* REF. 65 © (2006) Macmillan Publishers Ltd. All rights reserved. Panel **d** is reproduced, with permission, from REF. 67 © (2008) National Academy of Sciences.

These results provide a starting point for further analysis of the role of specific circuit interactions in the heterogeneous process of differentiation.

Coordinating gene expression. Cellular responses to external signals involve two steps. First, the external signal must be represented in the cell in the concentrations, states and dynamics of transcription factors. Second, these active transcription factors in turn regulate the expression of genes. By enabling researchers to follow changes in localization of transcription factors, movies have revealed a dynamic encoding scheme used in the response of yeast to calcium stress.

Cai *et al.* acquired movies of individual yeast cells in which the calcineurin-responsive zinc finger transcription factor Crz1 was fused to a fluorescent protein[74]. Crz1 is dephosphorylated by calcineurin in response to calcium. When dephosphorylated, it transits to the nucleus, where it can activate target genes. Movies of Crz1 revealed that a step change in extracellular calcium levels caused rapid stochastic bursts of Crz1 nuclear localization, rather than a steady shift in the otherwise static fraction of Crz1 molecules in the nucleus. These bursts continued throughout the movie, typically for about 10 hours. Careful analysis of these bursts at different levels of calcium showed that calcium controls the frequency, but not the duration, of these bursts. Thus, the cell encodes calcium levels using a frequency modulation system.

What advantage does frequency modulation encoding provide the cell? A basic problem for cells is how to coordinate multiple target genes so that they are expressed in fixed proportions across a wide range of expression levels. In bacteria, operons can perform a similar function for small groups of genes. How might eukaryotic cells, which lack operons, achieve coordination? Frequency modulation regulation enables Crz1 to coordinate the expression of its many target genes in fixed proportions across many levels of activity. This is because calcium effectively regulates the fraction of time Crz1 is active (nuclear localized) rather than the fraction of Crz1 molecules in the nucleus. Consequently, all target genes are expressed in proportion to nuclear localization burst frequency. As the frequency increases (at higher calcium), all target genes increase their expression by the same factor.

This general mechanism coordinates target genes even when their individual GRFs differ for factors such as affinity and cooperativity. Because nuclear localization dynamics are unsynchronized, and appear stochastic, this

Frequency modulation
A way to encode information about the frequency of events or oscillations. Frequency modulation is often contrasted with amplitude modulation, in which signals are encoded by varying the magnitude of a signal. In engineering applications, such as broadcasting, the frequency-modulated signal is typically periodic (oscillatory). In the example of Crz1 it is the frequency of discrete stochastically timed bursts that is varied.

basic principle of gene regulation could only be identified using movies. It will be interesting to see what role the strategy of frequency modulation regulation plays in regulation in other biological systems, from bacteria to multicellular organisms, as similar activity dynamics have been observed in diverse systems[75–78].

Conclusions and future directions

As the examples described above make clear, movies are revealing an unexplored world of interesting regulatory strategies, mechanisms and behaviours. As more systems are imaged in more ways, we anticipate many more interesting discoveries. In model organisms, circuits can be studied more systematically and with greater throughput to analyse interactions among many pathways or genes simultaneously. Microscopy systems with improved automation can facilitate this transition[79]. Most genetic circuits studied so far involve the stress response or differentiation in model organisms, but many other cellular processes, such as metabolism, are amenable as well. It will also be interesting to see whether movies can be used to study circuit behaviours in natural strains or species, in environments that more closely resemble natural conditions. For all of these techniques, an outstanding question is to what extent correlations, and even dynamic correlations, can provide sufficient information to infer regulatory interactions or distinguish between possible modes in which circuits could operate[96].

Synthetic genetic circuits that are engineered to implement novel biological functions[52,53] provide unique opportunities to study potential genetic circuit designs. Can such circuits be engineered to operate consistently in all cells? Conversely, can they be designed to mimic biological strategies that take advantage of heterogeneity? Movies provide a powerful means to address these questions.

Some limitations remain. First, despite spectacular work in diversifying the palette of fluorescent protein reporters[80,81], few distinguishable colours can be used routinely to tag multiple genes. Second, quantitation and tracking of gene expression in individual cells in movie data remains a time-consuming process that is usually optimized differently in each laboratory. This is often the rate-limiting step for using movies to analyse circuits. More general software tools are needed to expand the usefulness of these techniques. Third, the effects of cell–cell interactions remain difficult to study. Proximity of cells in movies can provide clues, but the multitude of potential communication mechanisms and channels that exist ensure that many possible interactions can influence cell behaviour. Chemostatic microfluidic systems are beginning to address some of these concerns[82,83]. Similarly, many systems grow in three dimensions (for example, biofilms), making quantitation challenging[84].

Despite these challenges, movies are providing unique insights into how individual cell behaviour results from specific genetic circuit architectures. Only movies can provide a direct view of genetic activities in individual cells — the quantities that we use in mathematical models — that are at best approximated and at worst completely misrepresented by population averages. Moving forwards, one can also anticipate the broader use of time-lapse microscopy to compare the dynamics of related circuits across species[85].

The examples described above provide a tantalizing glimpse of the kind of direct insights movies will provide into circuit behaviour. In particular, movie analysis of circuits has already begun to resolve the two seemingly contradictory views of cellular function that were introduced at the beginning of this Review: variability can be generated from certain circuit designs, such as those incorporating positive feedback, but circuit functions, such as differentiation, require variability in the form of stochastic noise.

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DATABASES
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
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FURTHER INFORMATION
Michael Elowitz's homepage: http://www.elowitz.caltech.edu
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