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Measurement of single-cell dynamics

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Populations of cells are almost always heterogeneous in function and fate. To understand the plasticity of cells, it is vital to measure quantitatively and dynamically the molecular processes that underlie cell-fate decisions in single cells. Early events in cell signalling often occur within seconds of the stimulus, whereas intracellular signalling processes and transcriptional changes can take minutes or hours. By contrast, cell-fate decisions, such as whether a cell divides, differentiates or dies, can take many hours or days. Multiparameter experimental and computational methods that integrate quantitative measurement and mathematical simulation of these noisy and complex processes are required to understand the highly dynamic mechanisms that control cell plasticity and fate.

The processes that control cell fate (Fig. 1) occur across varying timescales. A cell must first detect and integrate multiple extracellular signals. Early responses (such as calcium signalling) can occur within a few seconds or minutes of ligation of a receptor at the cell surface (Fig. 2a). The consequent second messenger signalling and protein interaction and modification cascades often involve feedback loops, with spatially compartmentalized interactions and reversible reactions. A crucial step is transmission of the signal to the nucleus (Fig. 2b), through the translocation of proteins and/or the controlled generation of modified nuclear proteins that can modulate gene expression. Multiple gene expression processes are altered as a result, including transcription (Fig. 2c), translation, and epigenetic processes such as chromatin modifications. This, in turn, alters the concentrations of specific proteins (and non-coding RNAs such as microRNAs)[1]. These changes then lead to new cell phenotypes that determine a cell's fate (for example, to undergo commitment to cell division) (Fig. 2d).

The integrated measurement of all components involved in these processes in single cells is not possible with current technologies, although in the past 30 years there has been a marked increase in the number of experimental tools that are available for carrying out single-cell measurements. Almost 20 years ago, it was argued that molecular biologists needed to collect more quantitative data if complex cellular processes were to be understood[2]. Genome sequencing has since provided access to the genetic database that underlies these processes. So far, however, the biochemical processes in a cell can be only poorly quantified, limiting the ability to resolve the dynamic molecular processes that underlie important cell-fate decisions such as differentiation (or dedifferentiation), cell division and cell death.

Cellular heterogeneity is a feature that is intrinsic to many cell-fate processes, including cell division, apoptosis[3] and the generation of induced pluripotent stem (iPS) cells[4] (see pages 704 and 713). Cell signalling[5] and transcription[6] are also surprisingly dynamic, often resulting in heterogeneous responses among cells. At the cell population level, this heterogeneity can be discerned only when the process of interest has been synchronized between cells (for example in studies of the cell cycle[7], the circadian clock[8], transcriptional cycles[9] and cell-signalling dynamics[10]). Often cell synchronization is neither possible nor desirable when studying physiologically relevant processes.

Even if all of the relevant molecular measurements could be made, the great complexity of the data makes it difficult to process, integrate and interpret the information. Intrinsic stochastic events, which are associated with small numbers of molecules and gene copies[11], generate 'noise'. This noise might need to be minimized by the cell, but it might also be used as part of the mechanism of decision-making[3].

Here, we review the experimental tools that can be used to measure different stages in the regulation of cell signalling, transcription, plasticity and fate in single cells (as summarized in Fig. 1). We also describe current methods for experimentally manipulating single-cell function and for improving experimental throughput by using microfluidic technologies. We then outline mathematical modelling tools that are available for predicting, quantifying and understanding these complex dynamic processes. Finally, we discuss how the integration of complex information and the iterative generation and testing of hypotheses are becoming increasingly important for understanding new roles for molecular dynamics in cell function.

Measuring early signalling events

Numerous receptors are present at the surface (and in the nucleus) of each cell. Ligation of a receptor activates one or more intracellular signalling pathways. Early responses to signals at the cell surface include the activation of ion channels and the release (or entry to the cell) of second messengers such as calcium. The development and application of the calcium-sensitive photoprotein aequorin[12,13], as well as improved chemical fluorescent sensors of calcium[14] (Fig. 2a), revealed that the frequency of calcium oscillations is one of the main mechanisms that the cell uses to encode information in a spatio-temporal manner[15,16]. From these observations, the important roles of molecular dynamics and frequency encoding of information in cell signalling were first characterized.

Studying the electrophysiology of ion channels in single cells has been facilitated by the patch-clamp method[17]. This technique allows researchers to record the currents passing through single ion channels, demonstrating the role of specific channels in cellular processes, such as action potential conduction. Recently, automated patch-clamp approaches[18] have allowed experiments to be carried out with considerably greater throughput. Calcium imaging and patch-clamp techniques have also been successfully integrated in many studies. For example, one such study used patch-clamp electrodes to impose specific patterns of calcium spike activity on differentiating neurons[19]. Subsequently, the maturing neurons produced inappropriate neurotransmitters, which did

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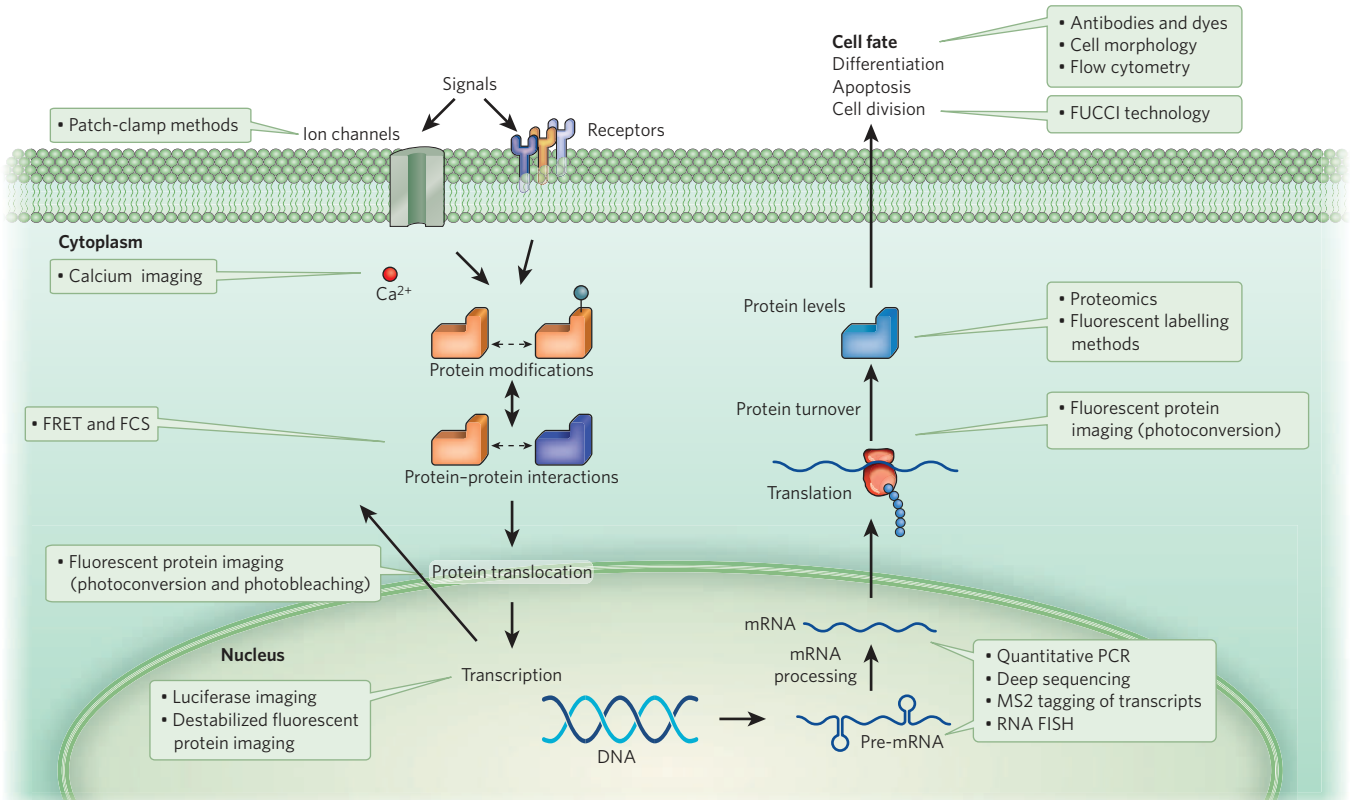


Figure 1 | Dynamic processes in living cells. The diagram summarizes the sequence of events from signal recognition to cell fate and indicates some of the measurement technologies used for quantifying these processes. The diagram recapitulates the order in which these processes are reviewed here, starting with signals at the cell membrane. Individual methods are further illustrated by examples of time-lapse analysis of cell processes in Fig. 2. FCS, fluorescence correlation spectroscopy; FRET, fluorescence resonance energy transfer; FUCCI, fluorescent, ubiquitylation-based cell-cycle indicator; mRNA, messenger RNA.

not match the corresponding cell identities as determined from marker expression profiles.

Measuring protein translocation

In 1994, it was discovered that green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* could be fused with other proteins in many cell types to form fluorescent fusion proteins[20]. This finding led to the rapid development of tools for tracking intracellular proteins[21], which resulted in considerable progress in analysing the spatiotemporal dynamics of signalling proteins in single cells.

Given the typical dimensions of a mammalian cell (10 μm in diameter), and given that protein diffusion in water is a rapid process (about 10 ms μm^{-1})[22], each protein molecule could traverse the entire diameter of the cell ten times each second. In practice, many proteins are restricted in their diffusion, although some proteins are also actively transported. The movement of fluorescently labelled proteins within a cell can be quantified with the help of time-lapse microscopy (Fig. 2b).

Rates of protein movement within and between cellular compartments can be measured by photobleaching or photoconverting a fluorophore on a protein in a specific region of the cell and then measuring the rate at which the fluorescence recovers or disperses. Variations of these techniques include fluorescence recovery after photobleaching (FRAP)[23], fluorescence loss in photobleaching (FLIP) and the use of photoconvertible fluorescent proteins[21] (Box 1). For quantitative analysis, diffusion in the *z* plane is difficult to accommodate; therefore, mathematically, these approaches are most suitable for flat cells (such as many adherent cell types). An alternative approach, fluorescence correlation spectroscopy (FCS)[24], measures the time that fluorescent molecules take to pass through the confocal volume of laser light, allowing the diffusion rates and absolute concentrations of fluorescent molecules to be calculated in specific cellular regions (Box 1).

Findings from fluorescent-protein translocation studies have recently indicated that, like calcium signalling, protein-based signalling (often through nonlinear dynamics) might exploit the timing of protein movement and protein modification to control downstream cellular events[10,25,26]. For example, fluorescence time-lapse imaging of single cells has shown that the transcription factors nuclear factor-κB (NF-κB)[5] (Fig. 2b) and p53 (ref. 27) undergo out-of-phase oscillations between the nucleus and the cytoplasm with a typical frequency of about 100 min for NF-κB and 5–6 h for p53 (see Box 2 for other examples).

Photobleaching experiments also led to another key discovery about the dynamics of intracellular processes: that the hormone-occupied glucocorticoid receptor undergoes rapid exchange between the nucleoplasm and the chromatin[28]. These and similar studies showed that the interaction of regulatory proteins with target sites in the chromatin is a more dynamic process than had previously been thought.

Measuring protein interactions and modifications

Protein–protein interactions have traditionally been assessed either *in vitro* by using purified proteins or by co-purification in biophysical assays (for example by immunoprecipitation of cell lysates) or in cells by using yeast or mammalian two-hybrid assays[29]. These assays do not take into account the physical separation of proteins within cells and give no indication of the dynamics of interactions.

To overcome this limitation, protein–protein interactions in cells can be studied by labelling candidate protein binding partners with compatible fluorophores and then measuring the efficiency of fluorescence resonance energy transfer (FRET)[30] between the labelled proteins (Box 1). Because FRET has an effective range of ~1–10 nm, interactions that occur over a longer range cannot be detected. Thus, FRET can only confirm that protein interactions are occurring but cannot exclude the possibility that they are not. Another technique, fluorescence lifetime

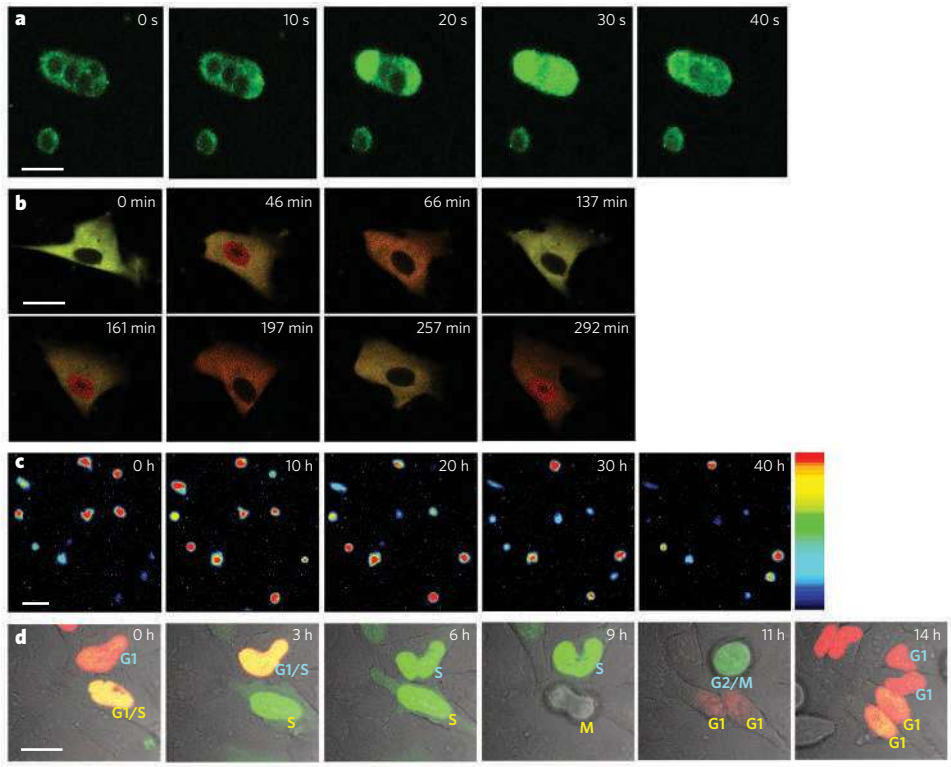


Figure 2 | Examples of time-lapse imaging of single cells. a, Early signalling events. Calcium imaging of pituitary cells (of the GH3 cell line) is shown after treatment with thyrotropin-releasing hormone. Fluctuations in the amount of calcium in the cytoplasm over seconds were visualized by using fluo-4 dye (green) (Supplementary Movie 1). Scale bar, 20 μm. **b**, Transcription-factor translocation. Fluorescent protein imaging of neuroblastoma cells (of the SK-N-AS cell line) treated with tumour-necrosis factor-α is shown. The protein RELA (which is a subunit of the transcription factor nuclear factor-κB) was fused to the fluorescent protein DsRed-Express (red). RELA oscillates between the cytoplasm and the nucleus of cells with a period of about 100 min. Concurrently, the RELA inhibitor IκBα, labelled with enhanced green fluorescent protein (green), shows cycles of synthesis and degradation that have an inverse phase to the cycles of RELA translocation[5] (Supplementary Movie 2). Scale bar, 20 μm. **c**, Transcription analysis. Low-light-level imaging of pituitary cells (of the GH3 cell line) expressing luciferase under the control of the promoter of the human prolactin gene is shown. The substrate of luciferase, luciferin, was added to the medium, and images were taken at 15-min intervals over hours. The colour scale indicates the range of light emission, from low (blue) to high (red). The cycles of transcription are heterogeneous across the cells[65] (Supplementary Movie 3). Scale bar, 50 μm. **d**, Cell division. Imaging of epithelial cells (of the HeLa cell line) by using FUCCI technology, over hours, is shown. Cells transiently express FUCCI proteins, depending on their differing stability at different phases of the cell cycle: G1 phase (red), S phase (green); G2 phase (reduced green fluorescence) and M phase (no fluorescent signal)[75]. Blue labels indicate the cell-cycle phase of a single cell in this image series, and yellow labels indicate that of another single cell. Each of these cells divides over the course of the experiment (Supplementary Movie 4). Scale bar, 20 μm.

imaging microscopy (FLIM)[31] (Box 1), has the advantage that the signal measured is independent of fluorophore concentration (a factor that can confound interpretation of FRET results). Recently, FRET donor proteins with improved single exponential decay rates[32], as well as non-fluorescent FRET acceptor proteins, have been developed[33]. As a result, it will now be possible to carry out multiplex (many simultaneous) measurements with FRET sensors by using time-lapse FLIM.

FRET has been exploited to develop several sensors based on intramolecular interactions. One of the earliest such fluorescent sensors to be developed was the Cameleon set of probes for sensing calcium[34]. Subsequently, probes for a wide range of intracellular processes have been developed[35]. A non-FRET-based approach can also be taken: one such technique makes use of circularly permuted fluorescent proteins[36], which, for calcium sensing, show a higher dynamic range than FRET sensors. These proteins are encoded by genetic reporters, so their advantage lies in their steady-state expression, which allows extended time-lapse measurements to be made. They can also be targeted to specific cell compartments and to particular cell types and tissues *in vivo*.

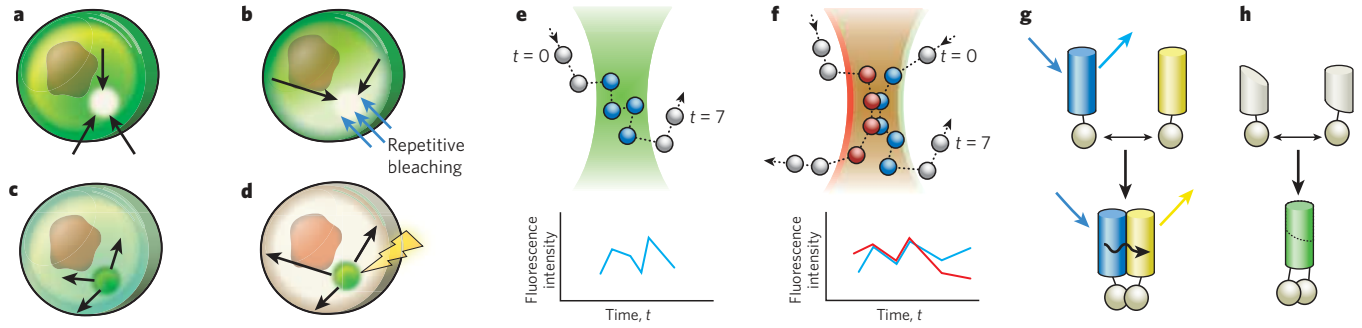
More quantitative methods for measuring protein–protein interactions in living cells are fluorescence cross-correlation spectroscopy (FCCS) and its variant known as image cross-correlation spectroscopy (ICCS)[37], both of which are based on FCS (Box 1). Recently devised techniques that increase the spatial resolution of light microscopy beyond the optical diffraction limit[38] (about 200 nm) might ultimately enable protein–protein interactions to be inferred through direct measurement of protein co-localization at molecular resolution.

Protein–protein interaction studies in single cells have often confirmed the physiological relevance of other biochemical studies of cell populations and other *in vitro* studies, and they have been particularly useful for studying the interactions of cell-surface receptors. It seems probable that single-molecule FRET studies will be increasingly important for analysing molecular dynamics and function[39].

Analysing transcriptional control in single cells

The regulation of transcription is central to cell-fate determination (see page 704). Transcription in single cells is measured directly, by measuring messenger RNA levels, or indirectly, by the imaging of non-invasive reporters such as firefly luciferase[40] or destabilized fluorescent proteins[41]. Several of these indirect techniques — luciferase imaging[42–46] (Figs 2c and 3), β-galactosidase imaging[47,48] and RNA fluorescent *in situ* hybridization (RNA FISH)[49] — provided the first evidence that transcription was more dynamic and stochastic than had been suspected. Carrying out RNA FISH on single fixed cells allowed the transcription burst size to be mathematically quantified in mammalian cells[50], and the dynamics of transcriptional pulsing in single cells[51] can be directly visualized by using a fluorescent protein fused with the coat protein of bacteriophage MS2. The fluorescent fusion protein binds to specific RNA stem–loop structures, which can

Box 1 | Methods for measuring molecular dynamics and interactions



Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP)
These techniques involve the photobleaching of fluorescent molecules within a specific region of the cell. FRAP involves recording the subsequent recovery of fluorescence as the fluorescent molecules repopulate the photobleached region (**a**, black arrows indicate the direction of fluorescent molecule movement)[23], whereas FLIP involves observing the reduction in fluorescence outside a repetitively bleached region (indicated with blue arrows), caused by the continual repopulation and destruction of fluorophores that enter the bleached region from outside (**b**). This allows information to be derived on the rates of movement of the fluorescent species. In inverse FRAP (**c**), cellular fluorescence in the whole cell area outside a small region is photobleached, and the subsequent decrease in fluorescence in the unbleached region (intense green area) is then recorded.

Photoactivation and photoconversion
These methods use derivatives of fluorescent proteins that can be switched on or off, or can change their fluorescence emission intensity (photoactivation) or spectrum (photoconversion), in response to light[21] (**d**). These approaches can provide similar data to FRAP and FLIP, but they can be used over longer timescales in 'pulse–chase'-type experiments to determine protein turnover rates because they do not involve fluorophore photobleaching.

Fluorescence correlation spectroscopy (FCS)
This technique measures the number of fluorescent molecules and the time that they spend in a diffraction-limited volume of light, by using a detector that observes a confocal region[24]. The trajectory of a single fluorescent molecule is shown before (grey, $t = 0$), during (blue, excited) and after (grey, $t = 7$) entering the illuminated region, with the measured fluorescence (blue line) during this time period shown below (**e**). From the correlation function that is produced, the rate of diffusion of the fluorescent species in **e** can be calculated, together with the absolute number of molecules in the confocal volume. By extrapolation, the concentration of the protein can be estimated for a given cellular compartment.

Image correlation spectroscopy (ICS)
Whereas FCS collects data from one or a small number of observation volumes, ICS analyses point-to-point fluorescence fluctuations from the multiple observation volumes present in raster-scanned laser confocal microscopy images. It has been developed for time-lapse imaging of single cells[37].

Fluorescence cross-correlation spectroscopy (FCCS)
This is a derivative of FCS, in which temporal correlations of the intensity fluctuations of two or more fluorophores are measured within a confocal volume (**f**). Blue and red trajectories show the movement of two transiently interacting molecules, from $t = 0$ to $t = 7$. Each molecule shows fluorescence when within the exciting confocal beam. The blue and red lines, below the diagram, show the fluctuations in fluorescence. This can be used to derive the binding relationships of different molecules through the correlation between the times that they spend together in the imaging volume.

Image cross-correlation spectroscopy (ICCS)
This method is an extension of ICS (equivalent to FCCS) that produces similar data but for protein–protein interactions[37].

Fluorescence resonance energy transfer (FRET)
This method measures whether two spectrally compatible fluorophores (blue and yellow in **g**; arrows depict excitation and emission), and therefore two proteins of interest, are in close proximity (1–10 nm)[30]. The fluorophores need to be in a favourable dipole–dipole orientation, allowing non-radiative transfer of energy from the excited donor fluorophore to the acceptor fluorophore. In this way, the binding interaction between donor and acceptor can be followed by determining the efficiency of this energy transfer, through measuring changes in the intensity of acceptor fluorescence emission or through an associated reduction in donor fluorescence (donor quenching). The interpretation of such intensity-based measurements is limited by experimental artefacts such as the relative concentration of the two fluorophores, signal cross-contamination and variations in excitation intensity. These limitations can be ameliorated by combining FRET with FLIM.

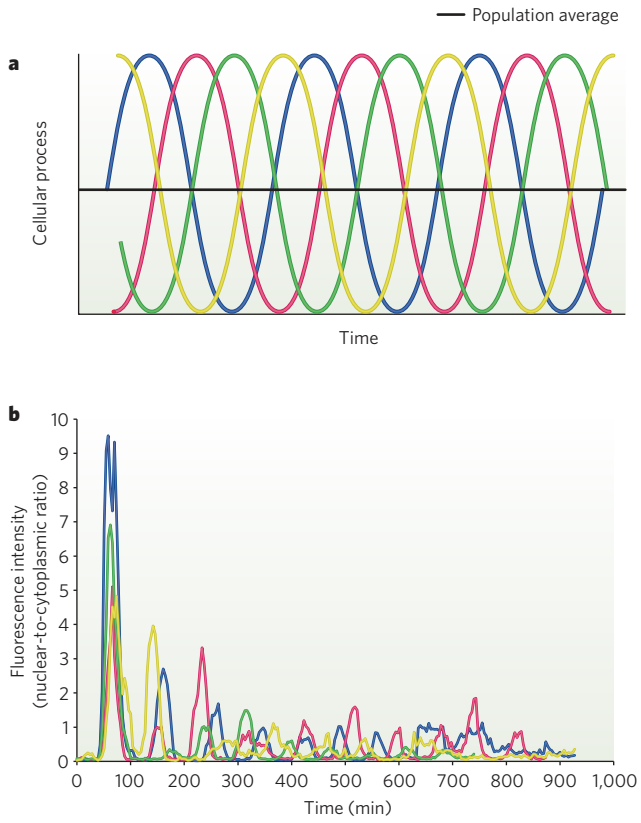
Fluorescence lifetime imaging microscopy (FLIM)
This method measures the rate of decay of donor emission[31]. Increased energy transfer (FRET) decreases this parameter. This rate of decay is unaffected by many of the factors that negatively affect intensity-based FRET measurements.

Protein-fragment complementation assay (PCA)
These assays[120] rely on the expression of two 'half' domains of a reporter protein fused to separate proteins. The reporter protein can be either a fluorescent protein, in the case of bimolecular fluorescence complementation (BiFC)[121] (**h**), or another measurable enzyme such as a luciferase[122]. If the two proteins interact, then the split domains of the reporter protein are reconstituted, and a signal is observed (green in **h**). PCA has a low background signal and compared with FRET has a greater tolerance for separation of the interacting proteins.

be engineered into any RNA molecule of interest, allowing fluorescent protein labelling of the RNA molecule in cells[52]. The dynamic nature of transcription may be important in the acute response of cells to signals.

Static (single time point) measurements (for example single-RNA counting in single cells[50]) have greatly aided the quantification of transcription in single cells. Multiplex measurement of gene expression in single cells can be achieved by using RNA FISH[53], PCR[54], transcriptomic analysis[55] or RNA-Seq[56]. Applying more sensitive sequencing methods to single-cell analysis is likely to have a large impact on the ability to analyse transcription initiation, as well as measuring transcription elongation and mRNA processing.

Box 2 | Oscillatory signal dynamics



Many cell-signalling and transcriptional processes show pulsatile, or even oscillatory, behaviour. If such processes occur out of phase in cells across a population, then techniques that measure biological parameters in the whole population of cells result in these dynamics being averaged out across the cell population and thus not being observable. This is illustrated in the schematic graph (**a**), which shows out-of-phase oscillations in a process occurring in single cells (each indicated by a different colour). Measuring this process in the cell population would result in a stable profile (black), the average across the cell population. Similar results are obtained from a real example (**b**). Translocation of NF-κB from the cytoplasm to the nucleus, and back, was imaged in single neuroblastoma cells (of the SK-N-AS cell line) after stimulation with tumour-necrosis factor-α[5] (see also Fig. 2b and Supplementary Movie 2). Data for four cells are shown. After the first peak of fluorescence in the nucleus, the cells show out-of-phase oscillations. In recent years, numerous key signalling and cellular processes have been found to show such pulsatile or oscillatory dynamics. Examples of such systems are listed in the table.

Cellular signal	Period length	Reference example
Calcium	Seconds to minutes	15
ERK2	~15 min	101
Transcription cycles	Tens of minutes	9
NF-κB	~100 min	5
Crz1 (similar to NFAT)	Random, pulses occur over minutes	25
Segmentation (Notch, WNTs, FGFs)	~90 min	116
STAT signalling	~2 h	102
p53	5–6 h	27
Cell cycle	~18 h	100, 119
Circadian rhythms	24 h	117, 118

ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; NFAT, nuclear factor of activated T cells; STAT, signal transducer and activator of transcription.

Another important aspect of the control of gene expression lies in the epigenetic organization of nuclear structure. Knock-in transgenic cells[57,58] or bacterial artificial chromosomes have been used to study the regulation of genes in their native chromatin context[59,60] (Fig. 3). Epigenetic modifications — DNA methylation and histone modifications — have recently been found to be more dynamic than previously suspected[61], and transcriptionally active genes are now known to be directed to spatially organized transcription factories[62], where coordinately regulated genes can be co-transcribed[63]. Chromatin immunoprecipitation studies of populations of tightly synchronized cells have suggested that organized cycles of transcription can occur at certain promoters[9]. There is therefore a need for improved approaches to relate the dynamic aspects of nuclear architecture (for example chromosome topology, and DNA and histone modifications) to gene expression and cell fate in single cells. It has recently been shown that cells must divide and undergo the associated DNA demethylation[64] for changes in gene expression that are associated with commitment of a cell to an induced pluripotent state[4].

The importance of stochasticity in dynamic and unstable transcription was uncovered by using single-cell RNA FISH, which showed stochastic switching between the expression of alleles in cells undergoing the transition from fetal to adult globin gene expression[49]. Some cells expressed unprocessed fetal globin transcripts while adult globin mRNA was present in the cytoplasm from a previous round of transcription. In another example, luciferase imaging studies showed dynamically stochastic transcription from an HIV-1 promoter[46]. More recent studies suggest that there is plasticity (that is, controlled dynamic variability) in expression of the gene encoding the hormone prolactin in the anterior pituitary gland[45,65], where a mixed population of cells produces a set of physiologically important hormones that are regulated acutely (Figs 2c and 3).

Measuring protein levels

Cellular plasticity depends ultimately on changes in protein expression levels. These changes are most commonly measured by assaying protein expression from just one (or a few) marker gene(s) and are often measured at only one (or a few) time point(s). The measurements are generally poorly quantitative and expressed only in terms of relative protein levels. Absolute quantification of protein levels in cells is a non-trivial task. Most assays rely on antibody-based detection of proteins in an extract from a cell population and are rarely calibrated with the purified protein of interest. Such assays include western blotting, enzyme-linked immunosorbent assays (ELISAs) and radioimmunoassays[66]. Epitope tagging[67], by contrast, avoids the complication of using many antibodies but at the expense of the tag possibly causing perturbations. Recently, elegant proteomic mass spectroscopy methods have been developed, allowing the absolute levels of many proteins to be measured in parallel[68]. None of these techniques, however, has the sensitivity required to measure protein concentrations directly in a single cell.

In single cells, immunocytochemistry, whether in conjunction with flow cytometry or microscopy, has often been used to assess the relative protein levels and gives an indication of the intercellular heterogeneity of the proteins. Microscopy can provide information about the intracellular distribution of the proteins, but these data must be back-calibrated to those from bulk-cell analysis in order to derive information about protein concentrations. This strategy ultimately relies on the quality and specificity of the antibodies used, the purity of the calibration protein, and the consistency of cell-sample processing. To measure the intracellular levels of proteins, cells generally need to be fixed, permeabilized or lysed, to allow access of the antibody to the antigen. These processing steps can lead to protein degradation, differential protein loss and/or destruction of the antibody-binding epitope(s). Despite these caveats, there are many elegant studies examining the relative abundance of proteins, their localization in cells[69] and their relative expression levels in different tissues and tumour types[70] (see also the Human Proteome Resource database, http://www.proteinatlas.org/intro.php).

For the future, one of the most promising approaches for localizing and counting specific individual macromolecular complexes in intact cells is visual proteomics. This technique computationally matches

structures in cryo-electron tomographs with high-resolution reference structures obtained by electron or X-ray diffraction[71].

An alternative method for single-cell protein quantification is to use genetic constructs from which are expressed fusion proteins with either a fluorescent protein[21] or another genetically expressed tag that can allow fluorescent marking of the protein of interest. In this context, engineered biarsenical ligands that bind to specific tetracysteine motifs[72] and the enzyme-based SNAP-tag system for protein labelling[73] have been used. The tag, however, can alter the properties of the original protein, and the tagged proteins are typically overexpressed from viral promoters. More relevant measurements can be made if the tagged protein is expressed from its native promoter, most commonly from a bacterial artificial chromosome[59,60] or knocked into the relevant genomic locus[57,58]. Fluorescence levels can be assessed by calibrated flow cytometry or fluorescence microscopy, and the absolute concentration of fluorescent proteins in specific cell compartments can be quantified by FCS[24] (Box 1).

When assessing cell phenotype, the level of specific protein markers needs to be measured accurately (for example, the level of stem-cell markers is important for accurately distinguishing pluripotent cells from differentiated cells[4,74]). Whether a cell is undergoing cell death or cell division is often assessed by using time-lapse morphology studies or by flow cytometric analysis of DNA content. More precise single-cell assays for these heterogeneous processes are required[3]. In this respect, fluorescent, ubiquitylation-based cell-cycle indicator (FUCCI) technology, which was developed recently, identifies cells at different phases of the cell cycle more accurately[75] (Fig. 2d).

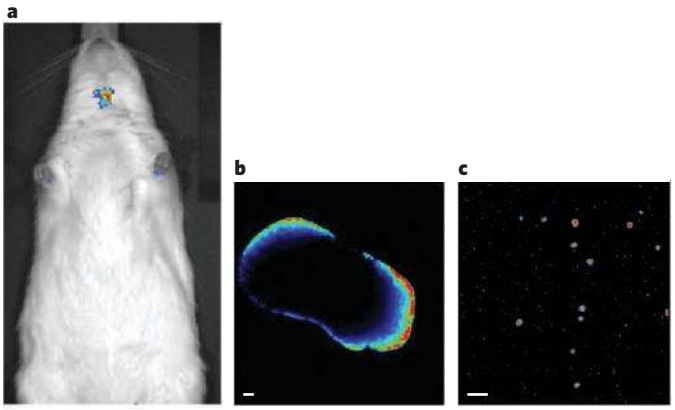


Figure 3 | Luminescent imaging *in vivo* and *in vitro*. **a**, A transgenic rat expresses luciferase in the pituitary gland under the natural control of the promoter of the human prolactin gene[60]. **b**, A cultured intact pituitary gland from a transgenic rat (**a**) shows localization of gene expression within the pituitary gland[65]. Scale bar, 100 µm. **c**, Single primary rat pituitary cells from disaggregated tissue, in culture, showing heterogeneity between individual cells, which show independent dynamic behaviour[65]. Scale bar, 100 µm. The luminescence intensity is shown in each image on a scale from low (blue) to high (red). These data show how individual cell heterogeneity must be considered when studying overall organ phenotype and whole-animal physiology.

Label-free measurements

Given the issues arising from the labelling or tagging of molecules, it would be advantageous to be able to analyse single cells in a manner that does not involve such modification. One such method is stimulated Raman scattering (SRS)[76], which uses two laser beams tuned to different frequencies. If the difference in frequency between the beams matches the vibration of specific chemical bonds, then one beam (the Stokes beam) will experience stimulated Raman gain, whereas the other beam (the pump beam) will experience stimulated Raman loss. These measurements of Raman gain and loss can be used to detect specific molecules in tissues and cells, which can be used to generate an image. A similar illumination regime generates a coherent anti-Stokes Raman scattering (CARS) signal, which can also be used to generate an image[77]. More recently, stimulated emission from naturally occurring chromophores such as haemoglobin[78] has also been used to provide image contrast. These techniques have great potential for cell imaging and molecular quantification, because they provide information about unmodified samples. At present, however, the spectral signatures of the chemical bonds must be relatively abundant to be detected, and the signal might arise from these bonds in multiple biochemical entities, making it difficult to interpret the data.

Manipulating single cells

To further study coordination of the cellular processes in single cells, it is often useful to examine the effects of experimental perturbations. These perturbations can take several forms, including the manipulation of gene expression and the cell-signalling environment. More specific changes can be induced by introducing chemical or optical switches into cells. As experiments to measure the relationship between early signalling events and cell fate become more complex and more physiological in their scope, the use of such tools for manipulating cells in a targeted manner will become increasingly important.

A relevant recent example of how cell fate can be manipulated is the induction of pluripotency in differentiated cells (see pages 704 and 713). The initial studies in which differentiated cells were reprogrammed involved the transfer of somatic-cell nuclei into enucleated oocytes[79,80], which led to animal cloning[81]. Recently, overexpression of a set of just four defined genes was found to generate iPS cells in mice and humans[4]. Moreover, it has now been shown that these iPS cells can be generated by delivering the proteins encoded by these four genes directly to the nucleus[82], indicating that the proteins are needed only up to a key commitment point. This procedure can therefore be used to generate pluripotent cells without requiring gene transfer.

The growing importance of RNA-interference technology[83] illustrates the usefulness of genetic perturbation in single-cell analysis. Numerous high-throughput genome-wide screens have used RNA interference to identify the functions of particular genes in single cells[84].

Another tool that is often used in combination with single-cell analysis is uncaging technology[85]. Caged compounds sequester molecules in a functionally inactive state that is reversed on irradiation of the cell. Initiating uncaging during an imaging experiment provides precise control of molecule release and allows particular cellular responses to be observed[86]. In a similar approach, a fused photoreactive domain has been used to generate a photoactivatable version of the protein Rac[87,88]. This technology allowed an exquisite temporal analysis of the function of Rac in living cells. Another strategy for controlling the state of a molecule of interest involves generating a fusion protein with FK506-binding protein, allowing protein stability to be regulated by exposure to the small molecule FK506 (ref. 89).

Microfluidic technologies

For single-cell studies, there is a considerable need for a platform that allows integrated multiparameter measurement and manipulation of cells. Microfluidic technologies are increasingly bringing new and improved protocols to single-cell analysis strategies. These technologies can be broadly divided into two classes: miniaturized, high-throughput and sensitive biochemical assays, with the scope for multiplexing analytical techniques that are difficult, or impossible, to link together by other means[90]; and single-cell trapping and manipulation systems that allow the long-term measurement of gene expression within gene networks[91,92] (Fig. 4). Microfluidic systems allow researchers to make temporally precise perturbations to the cellular environment (including generating static or dynamic gradients of molecules of interest). Such refinements can greatly simplify the interpretation of cell-signalling pathways and gene expression responses. Advances in combining elements from imaging analysis with biochemical assays, and with environmental control, promise to create a 'one-stop shop' for single-cell analysis — the often-cited 'lab-on-a-chip' approach.

Adapting traditional biochemical assays for single-cell analysis has proved problematic, because the true signal is often swamped by sample-handling variability and contamination when the raw material from single cells is diluted to microlitre volumes. Such problems can be overcome by using microfluidic devices, which comprise an interlinked series of channels, reaction chambers, microvalves and sample read-out devices, all of which are physically integrated on a microfabricated solid substrate. They allow reaction volumes to be scaled down to nanolitres or picolitres, improving the speed, sensitivity and throughput of the biochemical assay and reducing the reagent consumption.

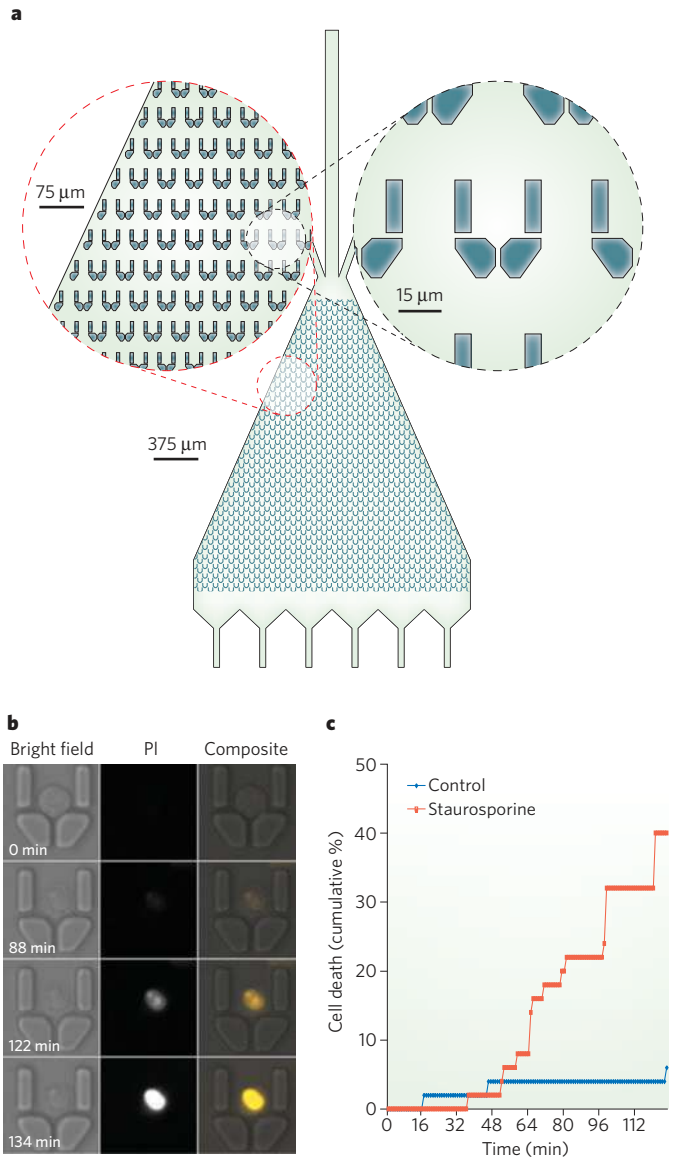


Figure 4 | Example of a microfluidic device for single-cell manipulation and long-term observation. a, The diagram shows a cell-trapping chamber containing 440 individual cell 'micro-jails'. The total volume of each chamber is less than 20 nl (a single chamber is shown in the magnification on the right). Suspensions of single cells are flowed through the chamber from the top such that individual cells become trapped in the micro-jails, facilitating their long-term observation. **b**, A promyelocytic leukaemia cell (of the HL-60 cell line) trapped in a micro-jail is exposed to staurosporine, an inducer of apoptosis. The medium contains propidium iodide (PI), a fluorescent dye that is normally excluded from viable cells. Over time, the PI-based fluorescence steadily increases, indicating the dynamic progression of the cell into apoptosis. Composite images of the bright-field and fluorescence micrographs are shown on the right. **c**, The cumulative percentage of cells dying over time is plotted for independent micro-jail arrays. Simultaneous monitoring of multiple micro-jails demonstrates the use of the microfluidic device to show the stochastic nature of the apoptotic process, as demonstrated by the varying incidence of cell death in the cell population over time (despite cells being exposed to the same environmental conditions). Such microfluidic devices have potential for drug-screening studies, because they allow the controlled delivery of candidate drugs. (Figure reproduced, with permission, from ref. 92.)

The features of microfluidic devices have paved the way towards complete *de novo* genome sequencing from a single template copy. Using a microfluidic Sanger sequencing procedure, and refinements in DNA capture and nanolitre-droplet encapsulation of individual DNA template molecules, accurate sequence reads were generated from 100 attomoles of template[93]. This is sensitive enough to allow the sequencing of single templates. This technology is likely to be especially useful for analysing somatic variation between individual cell genomes.

The inherent advantages of microfluidic technologies have also been exploited for single-cell gene expression analysis. Through integrating single-cell trapping, mRNA extraction and reverse-transcription PCR steps on a single chip, steady improvements in efficiency have led to the profiling of almost the complete transcriptome (corresponding to ~5,000 genes) of individual neural progenitor cells[94].

In addition to biochemical assays, microfluidic devices have been adapted to the imaging analysis of gene expression. Being able to confine and manipulate single cells is a great improvement for long-term imaging experiments, for which various designs of channel, microjail or microwell arrays have been used[91,92] (Fig. 4). After cells have been isolated, the environmental conditions are often manipulated to change the steady-state conditions or to establish (or alter) concentration gradients. Cellular responses such as chemotaxis, intracellular signal transduction and transcriptional regulation have been measured in this way, as has the stability of transcripts[95–97].

Model-led data integration and analysis

The quantities of experimental data and the number of reactions that regulate cell fate pose a major challenge to understanding cellular plasticity. Mathematical modelling and model-based data analysis are required to understand the behaviour and design principles of complex systems that show nonlinear behaviour in space and time (for example patterns, oscillations, switching and stochasticity). Modelling is also crucial for processing, integrating and interpreting complex high-dimensional data sets. It is therefore key for predictive biology and for rational experimental design[5,98].

Time-lapse measurements of single cells are ideal data sets from which to develop dynamic mathematical models. Deterministic mathematical models (for example using differential equations) can accurately simulate dynamic cellular subsystems such as the circadian clock[99], the cell cycle[100] and many signalling systems[10,101,102]. Cellular heterogeneity, however, is a common problem, and deterministic models cannot accurately simulate population-level or single-cell data unless the cells are relatively synchronous. Deterministic models basically assume that all cells are the same, which has the same outcome as experimental measurements of cell populations, which essentially average the data. Single-cell data are fundamentally noisy and can be fitted to deterministic models (for example by least squares) in only a limited set of circumstances. Therefore, stochastic models often need to be used in combination with deterministic models, in order to take into account cell-to-cell variation and noise, as well as to explain cellular heterogeneity[10]. For example, analysis of the noise structure (incorporating intrinsic and extrinsic noise) has been shown to be important in recent studies of the stochasticity that occurs in transcription and translation[11,50].

Stochastic simulations often use the Gillespie algorithm and its derivatives[103]. In this approach, a simplified mechanistic description of a biological system uses individual molecular reactions, which are assumed to have exponentially distributed half-lives (waiting times). Using stochastic differential equations is a powerful alternative. However, to be applicable to the modelling of real cellular systems, these equations require a minimum number of molecules to be present in the system. Also, the general mathematical theory for these approaches is underdeveloped for application to the high-dimensional and nonlinear systems found in cells.

Models can be broken down into those concerned with temporal or spatial aspects of cellular dynamics. Many studies of stochastic transcriptional dynamics use measurements from multiple single cells at a single time point. Dynamic stochastic models allow temporal dynamic information to be deduced from this static spatial information. Nevertheless, temporal aspects are best probed by direct time-series measurements, for example by using transcriptional reporters and fluorescently tagged proteins. Sophisticated algorithms have been developed to fit such data to models, and these algorithms can therefore provide estimates of hidden variables, for which data are unavailable[104]. For example, reporter genes provide an indirect measure of transcription, and the level of reporter activity depends not only on the rate of transcription (which is the parameter of interest) but also on the rate of RNA processing, the stability of the mRNA, the rate of translation, the formation of functional proteins (for example fluorescent protein formation) and the stability of the proteins. Each of these parameters needs to be taken into account to estimate transcription rates and to compare the results obtained with different reporters (which have different properties)[104]. The use of such algorithms together with techniques that directly probe mRNA levels in cells is likely to be particularly powerful.

Modelling is also important for the analysis of spatial data in single cells. For example, FRAP and FCS time-series data often need to be fitted to a model to deduce accurately the rates of protein translocation, diffusion and binding[23]. Spatio-temporal models have also been used to analyse the dynamics of fluorescent markers and to relate them to spatial physiological events such as pattern formation, cell movement and architecture reorganization[105].

As the complexity of the processes being studied grows, there is an increasing need for tools to analyse models[106] and for techniques to optimize or guide experimental design. Much of the current activity in this field relies on unguided simulation, but more sophisticated approaches use searching algorithms[107] that allow iterative improvement in the accuracy of simulations. There are promising approaches based on the Fisher information matrix, which allows rational identification of which variables to measure experimentally and what time resolution is required. However, these approaches require a model for the system to be developed and, at present, are local in parameter space and do not account for the information that is contained in the noise structure.

All of these models need to be in a common format so that they can be more easily understood and integrated together into larger models. For example, the Systems Biology Markup Language (SBML) is a machine-readable programming language that is based on Extensible Markup Language (XML) and is used for representing models of biological processes, including signalling and metabolic networks[108]. Data standards are also being developed for many types of 'omics' data. Time-lapse image analysis of living cells offers a particular challenge because the automated tools that are available are limited[109,110]. In systematic imaging screens for genome function[84], enormous quantities of image data can be generated, up to hundreds of terabytes of data from a single experiment. To manipulate and analyse such a large amount of data, automatic image handling becomes essential[111]. To this end, the development of common open access data standards for imaging[112] and other data types is an important priority.

Conclusions

The diverse technologies used for single-cell measurements reflect the complexity and variety of the processes that need to be probed if we are to understand the basis of cellular plasticity. There remain many areas where the technologies that are available have limited the ability to make accurate measurements of important processes, including the quantification of protein–protein interactions, post-translational modifications and the absolute levels of key proteins. The challenge is that the assays must be non-invasive to report on the dynamics of these processes in single living cells.

Cellular decision-making depends on the integration of single molecular events, as well as on interactions between cells. Averaging the molecular events within single cells can obscure dynamic single-molecule information in much the same way in which cell-population data mask the behaviour of individual cells. Isolated cells *in vitro* lack the correct environment that organizes and directs their behaviour *in vivo*. It is therefore important to consider how cellular plasticity is affected by regulation across biological scales. Several new fluorescent tomographic technologies now allow improved measurements of biological processes *in vivo*[113,114].

Populations of cells are characterized by a level of heterogeneity both *in vitro* and *in vivo*. Many signalling systems generate nonlinear dynamics through systems-level feedback. Observed cellular heterogeneity may be due, in part, to unsynchronized dynamics in single cells, possibly involving oscillatory processes[26]. Oscillations have been observed in key signalling pathways, including those that involve calcium[15,16], the transmembrane receptor Notch[115], the protein kinase ERK2 (ref. 101), and the transcription factors STAT3 (ref. 102), NF-κB[5], p53 (ref. 27) and yeast Crz1 (ref. 25) (which is similar to mammalian NFAT). Other processes such as the cell cycle[100], segment formation[116] and the circadian clock[117] are also oscillatory systems with varying periods. In most cases, oscillations have been shown to be (or are thought to be) masked at the population level by cellular heterogeneity, although in the case of the circadian clock this heterogeneity is overcome by intercellular communication[118] or by entrainment by external signals such as light or temperature. There is growing evidence for the importance of an oscillator's frequency in controlling downstream biological events[10,15,16,25,26]. It is now crucial to further develop experimental tools and mathematical models to understand better the roles and functional integration of these different processes. It will be interesting to explore how biological robustness is achieved within cell populations, despite heterogeneous and dynamic single-cell behaviour.

A key challenge is to understand the systems-level control of long-term cell-fate decisions. For instance, commitment to apoptosis is often extremely heterogeneous among cells in a population[3] and may take several days (indicating the presence of time-delayed and stochastic steps in the process). In the cell cycle, apparently irreversible transitions are regulated by systems-level feedback[119]. During development, differentiation takes place in a spatially and temporally organized and robust manner, as is also the case for segmentation (which involves multiple interconnected levels of feedback regulation)[116]. Do the seemingly opposite processes of reprogramming (during nuclear transfer or iPS-cell generation) and differentiation occur through the reversal of the same mechanisms and intermediate cell states? In addition, does the low efficiency of iPS-cell generation result from a low stochastic efficiency of the process, or is it caused by just a few 'elite' cells being capable of becoming pluripotent?[74] The former hypothesis is favoured at present, and there is considerable interest in understanding the stochastic processes that underlie these cellular decisions. In this way, there is an increasing need for single-cell analysis of molecular and cellular dynamics, coupled to an integrative and interdisciplinary systems-biology approach. This seems to be the most promising way to understand these important cellular decision-making processes. ■

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