REVIEW

High content imaging for monitoring signalling dynamics in single cells

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Abstract

All living cells are sensors of their environment: they sense signals, hormones, cytokines, and growth factors, among others. Binding of these signals to cell surface receptors initiates the transmission of messages along intracellular signalling pathways through protein–protein interactions, enzymatic modifications and conformational changes. Typically, the activation of signalling pathways are monitored in whole populations of cells, giving population average measures, often using experimental methods that destroy and homogenise the cell population. High content imaging is an automated, high-throughput fluorescence microscopy method that enables measurements of signal transduction pathways to be taken from live cells. It can be used to measure signalling dynamics, how the abundance of particular proteins of interest change over time, or to record how particular proteins move and change their localisation in response to a signal from their environment. Using this, and other single cell methods, it is becoming increasingly clear that cells are in fact very variable in their response to a given stimulus and in the quantities of cellular components they express, even in clonal (isogenic) cell lines. This review will discuss how high content imaging has contributed to our growing understanding of cellular heterogeneity. It will discuss how data generated has been combined with information theoretic approaches to quantify the amount of information transferred through noisy signalling pathways. Lastly, the relevance of heterogeneity to our understanding and treatment of disease will be considered, highlighting the importance of single cell measurements.

Introduction

Every cell in every tissue in the human body needs to be able to sense hormones, cytokines, growth factors and other signals in its environment in order to make appropriate decisions. Binding of such signals to cell surface receptors initiates signalling cascades, involving protein-protein interactions, enzymatic modifications and conformational changes, and many such signalling pathways are utilised by multiple types of receptor, in different compartments or regions of the cell. Signalling is dynamic; the abundance of signalling proteins and their localisation changes over time as the signal is transmitted, and this temporal aspect is important for the cell as a clock to activate different processes at different time points. Early signalling (seconds-minutes) can stimulate the release of calcium from intracellular stores to activate calcium-sensitive effectors, such as calmodulin and protein kinase C (e.g. G_q protein-coupled receptors, angiotensin II type 1 receptor or gonadotropin-releasing

hormone (GnRH) receptor), whereas gene transcription could be stimulated after several hours, and apoptosis or cellular senescence after several days or more.

In the laboratory, cell signalling can be observed in cell lines or primary cells in culture using western blotting to monitor the change in abundance of a protein of interest, or the change in level of post-translational modification it exhibits, such as phosphorylation, ubiquitination. A change in gene transcription downstream of signalling can be observed using RT-PCR to measure the amount of transcript present. Western blotting and RT-PCR require a near- confluent population of cells from one or two wells of a six-well plate, and these cells need to be homogenised to extract the protein or RNA, breaking cell membranes and disrupting compartments. An alternative is to use widefield or confocal fluorescence microscopy, which preserves the cell ultrastructure and enables signalling processes to be observed at the whole-cell level. Cells can be engineered or transfected to express a fluorescent protein; they can then be fixed, enabling detection of a protein of interest with subsequent antibody staining and fluorescent secondary antibody, or monitored live, where the movement or change in abundance of a protein can be observed over time in response to a hormone, cytokine or other stimulus. Traditionally, a researcher sits in a dark room at a fluorescent microscope for extended periods to manually capture images. For fixed cells, this may be choosing and capturing one field after another, or for live cell imaging, this may be following the trajectory of one or a small number of cells at a time.

Standard widefield and confocal fluorescence microscopes can be fitted with an automatic stage to make field selection less biased and more reproducible, and increase the number of fields that can be imaged, but when consistently large numbers of cells, fields and wells in multi-well plates are needed to be imaged with reliably even light illumination and fast auto-focussing, then is the time to invest in a high content imaging system.

Images can be analysed using appropriate software to quantify signalling in terms of the change in abundance or localisation of a particular protein of interest, either in fixed cells or over time, but even here, as with western blotting and RT-PCR, data has been traditionally reported as average responses for a whole cell population. It is only in the last ten years that computing power and data storage capabilities have improved sufficiently for us to monitor signalling in single cells, and together with single cell 'omics technologies' (genomics, transcriptomics, metabolomics, epigenomics, proteomics) (Chessel & Carazo Salas 2019), investigations have revealed that individual cells are actually very variable in terms of protein expression and response to a given stimulus, even in a clonal (isogenic) population (Elowitz *et al.* 2002, Tay *et al.* 2010). This review will discuss how high content imaging has contributed to our understanding of this cellular heterogeneity and its relevance to hormone signalling, in particular using examples from my own work, and the broader relevance of heterogeneity to our understanding and treatment of disease.

High content imaging

The term 'high-content' has been in use for over 20 years (Giuliano & Taylor 1998), and it denotes the multi-parametric nature of the data that this method can yield. It enables microscopy to be performed at scale, in a reproducible, unbiased manner. Cells are typically grown or seeded into 96-well or 384-well black-walled cell culture microplates, enabling multiple conditions, different drugs or other treatment regimes, at different time points, to be monitored in a high-throughput and standardised manner (Fig. 1). Such experiments are ideal for use in drug discovery pipelines for compound screening, and are frequently used in this way (Bickle 2010). Cells can either be treated and fixed prior to imaging or imaged as 'live cells' within the high content imaging system. These two approaches, whilst related, can reveal different aspects of the underlying signalling pathways, and each has their own limitations and their own challenges.

Fixed cell experiments offer the most scope in terms of experiment size, the number of plates and wells being limited only by the researcher's technique or stamina! Two 96-well plates treated concurrently tends to be reasonable, with not less than 5 min between treatment steps. Experiments of this nature can also be more time-consuming, not only in terms of tendency for a larger experiment size, but also in terms of the additional antibody-staining and washing steps required. Once fixed and stained, however, plates can be imaged when convenient over the next couple of days, provided the cell plate is protected from the light in a refrigerator or cold room to prevent signal degradation. Notably, robot arms are available that can perform all of the fixing and staining steps, and even load the plate from a plate hotel if needed. Fixing of the cells is usually carried out using paraformaldehyde or methanol, blocked with normal goat serum, BSA or milk powder, and stained with primary and fluorescent secondary antibodies following usual immunocytochemistry protocols before placing the microplate into the system for imaging. Alternatively, or as
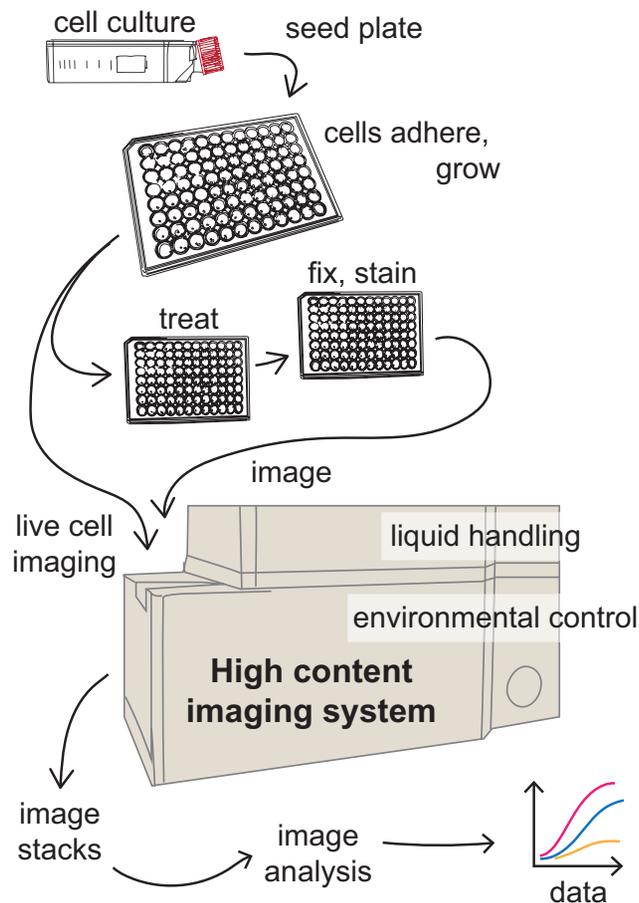
Figure 1

High content imaging workflow. Cells are typically grown in cell culture flasks and seeded into black-walled multi-well plates optimised for fluorescence imaging at a specified density. After a specified period of growth, the cells can be stimulated with hormones, drugs or other treatments at a range of concentrations and time periods, either for live cell imaging inside the high content imaging system or at the lab bench before fixing, (immuno-)staining and then imaging. The image stacks are subsequently transferred to another computer for image analysis and data processing.

well as, cells for fixed cell experiments can be engineered to express a protein of interest conjugated to a fluorescent protein, as for live cell imaging (discussed subsequently). Up to four fluorescent channels can typically be used during imaging, making multiplexing of several assays (e.g. in green, red and far red channels – provided that appropriate tests have been carried out to rule out bleed between channels), and staining is usually completed with a blue nuclear dye, 4′,6′-diamidino-2-phenylindole (DAPI) or Hoescht 33342.

Difficulties with fixed cell assays come from moving the cell plates into and out of the incubator for treatment steps, and the researcher should pay attention to their technique to ensure consistency between experiments,

for example is the timer started when the treatment is applied or when the plate is back in the incubator? It is possible to carry out time course experiments using fixed cell assays, although it will be different cells that are imaged at different time points, rather than the same cells monitored over time.

Live cell imaging, on the other hand, can track the same cells at specified time points over time, but there is always a balance to be struck between a short time interval between datapoints and number of fields and/or wells that can be imaged, since the time the objective takes to move and re-focus at each field and for each fluorescent channel needs to be considered. Antibody staining is not appropriate for live cells as it requires that the cells are fixed, and so the fluorescence required for imaging needs to be engineered (transfected or transduced) into the cells in the form of a fluorescent tag conjugated to the protein of interest. In addition, or instead of, some fluorescent dyes can work well in live cells (e.g. the change in intensity of the CellEvent caspase 3/7 green fluorescent dye can be monitored in live cell nuclei as a robust apoptosis assay (Garner *et al.* 2018)), but it should be noted that some dyes that work well for live cell imaging do not work well for fixed cells. Some dyes can be bleached if applied prior to fixing, particularly when fixing using methanol.

Trials during live cell imaging come in the form of keeping the environment constant, and we have found that in particular temperature fluctuations can be an issue. A heated stage is therefore indispensable for live cell imaging and should always be allowed as much time as possible to reach a stable temperature before imaging begins. High content imaging systems can include an environmental control chamber to stabilise the humidity and to enable CO_2 delivery, although we have found these not to be essential for any cell lines we have encountered so far. The CO_2 can be buffered with an appropriate buffer (Garner *et al.* 2017). On-board liquid handling systems to deliver drugs or other treatments are invaluable when cell responses are fast, for example for calcium imaging (Armstrong *et al.* 2009). Where cell responses are slower, as is the case for NFAT-EFP translocation (see subsequently), imaging can be performed in sections, with imaging paused during hormone or drug addition or washing steps. However, this itself brings issues in stitching image stacks across sections to enable image analysis software to monitor the same cell throughout the experiment (we wrote a MatLab script to stitch image stacks together in Garner *et al.* 2017). As with any microscopy, adherent cells that are large, flat and don't move around much when treated are ideal for high content imaging, but with improved high content confocal systems, it is

possible to develop assays for three-dimensional cultures and spheroid analysis.

Several companies manufacture high content imaging systems (Mattiazzi Usaj *et al.* 2016), and most offer both wide-field and confocal imaging models. Deconvolution methods are increasingly being offered as standard during the imaging process, enabling improved contrast to be gleaned in images, so improving the quality of the data that can be obtained from them, but using lower excitation power to avoid artefacts that can be associated with photodamage and phototoxicity in live cells. Notably, this limits the systematic variability in image acquisition. With improvements in image quality have come improvements in the speed of imaging, such that it is now possible to scan a 96-well plate in two fluorescent channels within 90 seconds.

It is only within the last 10 years that technology has progressed sufficiently to enable the analysis of single cells (Bickle 2010). The development of larger camera chips that can generate larger images of higher resolution and cameras that can re-focus fast enough, has been accompanied by improved computing power for image processing, networking technologies to move large amounts of data between computers and data storage facilities, which have themselves improved capacity to store large amounts of data.

Most high content imaging systems can be purchased with their own proprietary image analysis software, although other, open-source image analysis packages are available, for example, CellProfiler (a thorough discussion of this and other software packages is given by Eliceiri *et al.* 2012). The first step in image analysis is segmentation, in which individual cells are identified by computer algorithms, usually through recognition of the cell nuclei (Handly *et al.* 2016). From here, the cytoplasm, or regions within it, can be identified as staining permits. Delineating the plasma membrane can be particularly tricky without a specific plasma membrane stain, and it is, therefore, more beneficial to sample the cytoplasmic intensity using a ~3 μm collar, where a region boundary is drawn 3 μm outside of the nuclear boundary. Once identified, the amount of fluorescent signal in each region (i.e. nucleus, cytoplasm or other region defined and detected using an appropriate fluorescent stain) can be quantified, together with the size and other characteristics of each region, making it possible to track changes in response to drugs or other treatments in different parts of individual cells. Such image analysis software will generate a large amount of data, providing information about every cell in the experiment (defined by their X-Y co-ordinate in a field),

as well as data averaged across a field and across a well. A typical experiment might have 150–400 cells per field, typically four fields per well and two to three or more wells per condition per experiment, depending on the amount and type of data required. For some types of experiment, it might be sufficient to display the average response across a whole population; others may require the fine detail that can only be obtained from thousands of single cell responses, for example in experiments sorting cells based on the expression of a particular protein *in silico* (as for Ptpn1 (protein tyrosine phosphatase non-receptor type 1, PTP1B) expression in Garner *et al.* 2018) or for experiments investigating information transfer (see the 'Information transfer' section).

The most important indicator as to whether a high content screen or experiment will be a success is the quality of the assay. Automated microscopy enables changes in object localisation, intensity, texture or shape to be observed (Rausch 2006), and in theory, it is possible to configure an algorithm that will identify any feature of a cell that can be identified by eye, making many kinds of morphological assays, neurite outgrowth or tube formation possible (Bickle 2010). We have found particular success in developing assays that involve the nucleus, it being so straightforward to define. Even better for monitoring signalling dynamics are assays involving nuclear translocation, where the reduction in signal in one compartment is accompanied by an increase in signal in the nucleus (or vice versa). One such assay is the nuclear factor of activated T cells (NFATC1) tagged with emerald fluorescence protein (NFAT-EFP) nuclear translocation assay for monitoring calcium signalling, for example downstream of G_q-coupled receptors (Armstrong *et al.* 2009, Garner *et al.* 2017). Where a nuclear translocation event is not apparent as a readout for a signalling pathway, it may be possible to develop synthetic reporters that report the output of a pathway as a translocation event. An example of synthetic reporters of this nature are the kinase translocation reporters developed by Regot and colleagues, which couple a kinase recognition sequence with a nuclear export signal to translate kinase activation into nuclear export (Regot *et al.* 2014). Adequate controls are essential for the development of all types of assay. For nuclear translocation assays, positive and negative controls are necessary to define the dynamic range of the assay, as well as a means of identifying and removing the background fluorescence from calculations of nuclear-to-cytoplasmic (or cytoplasmic-to-nuclear) ratios. A particularly good review of assay development for high content imaging is given by Moreau & Gruenberg (2016).

Cellular heterogeneity and noise

As technology has advanced, the examination of single cells has revealed that even within a clonal (isogenic) cell population, much variation exists between cells in terms of response to stimuli and the expression of cellular components (Elowitz *et al.* 2002, Tay *et al.* 2010). It is becoming clear that examining only average responses across whole cell populations ignores the idiosyncrasies of individual cells, and can therefore suggest that the relationship between stimulus and response is much more clear cut than it might be in reality (Brennan *et al.* 2012). A normal distribution of response is assumed, but this is rarely the case. More usually, discrete subpopulations of responders are present, which give rise to multimodal distributions (Bickle 2010).

It might seem strange to think that the amount of protein produced by a particular gene varies from cell to cell, but put another way, how might a cell precisely measure the amount of protein present to an absolute cut-off value? Rather, noise, defined as the standard deviation divided by the mean, is present throughout the system, and sources of noise can be considered to be 'intrinsic' or 'extrinsic' (Elowitz *et al.* 2002). The expression of each gene is regulated by the concentrations, conformations and localisations of components of the protein synthesis machinery, polymerases and regulatory proteins, and any fluctuations in these correspond to fluctuations in expression of the gene under interrogation. These variations in cellular components that regulate gene expression have been defined as the extrinsic noise (Elowitz *et al.* 2002).

By contrast, intrinsic noise comes from local temperature fluctuations and random molecular diffusion, the nanoscopic events that govern which reactions occur and in what order. Intrinsic noise is generated by stochastic (random) promoter activation, promoter deactivation, and mRNA and protein production and decay (Kaern *et al.* 2005). Intrinsic noise has been described as the extent to which the expression of two identical copies of a particular gene, in the same intracellular environment, will fail to correlate (Elowitz *et al.* 2002). No matter how precisely the levels of the regulatory proteins are controlled, intrinsic noise will limit the accuracy of gene expression.

It is generally believed that cell–cell variability is more strongly linked to changes in the number of mRNA molecules than to the number of protein molecules (Kaern *et al.* 2005), and models of stochastic gene expression have predicted that intrinsic noise increases as the amount of gene transcript decreases (Elowitz *et al.*

2002). It has been shown that the least abundant proteins exhibit the greatest variability in their expression, and it is understood that this variation arises from the stochastic production and degradation of mRNA molecules that exist in very small numbers (1–2) per cell (Newman *et al.* 2006). Moreover, any cellular component that suffers intrinsic fluctuations in its own concentration will act as a source of extrinsic noise for other components with which it interacts (Elowitz *et al.* 2002).

Intrinsic noise is unpredictable and adds uncertainty at all time points, independently from one another (Selimkhanov *et al.* 2014). By contrast, extrinsic noise produces fluctuations that are constrained by the signalling network within certain bounds by positive and negative feedback mechanisms. In this way, the expression of proteins that function in the same pathway are co-regulated and correlate with one another in single cells (Sigal *et al.* 2006).

Just as we are accustomed to considering only population average responses, so too do we have a reliance on assessing protein function based on what happens to a population response if the expression of a particular gene of interest is reduced through gene knockdown or silencing approaches (e.g. RNAi or CRISPR), or protein expression is increased by plasmid transfection or viral transduction of an exogenous gene. It is notable then, in light of the discussion above of noise being increased when gene transcript levels are decreased, that unless from the experimental methods it can be verified with certainty that complete removal of transcript has been achieved (i.e. through clonal selection), any incomplete reduction in mRNA will result in an increase in noise. Similarly, for protein over-expression, an increase in noise is inevitable. Using fixed, single cell imaging with fluorescent antibody staining of a protein of interest, it can be possible to determine whether, and to what extent, cellular heterogeneity can be attributed to the knockdown or overexpression of the protein of interest.

Information transfer

In recent years, the application of information theoretic approaches to the study of signalling pathways has enabled us to learn about how cells respond to extracellular signals in a different way. In particular, mutual information (MI) treats a signalling pathway as a noisy communication channel, employing statistical methods originally developed in the communications industry for assessing information transmission along telephone lines. It is calculated from hundreds or thousands of individual

cell responses that have been recorded in response to a discrete range of stimulus values. Noise can restrict the transmission of a signal through a communication channel (Cheong *et al.* 2011), with intrinsic and extrinsic noise having different effects (Selimkhanov *et al.* 2014), and together both sources limit the 'power' that a cell has to alter its gene expression (Tkačik *et al.* 2008*a*). MI takes account of this noise to measure the quality of the inference (or 'prediction') of the signal from the response (Garner *et al.* 2016), and is defined as the uncertainty about the environment that is removed by signalling (Brennan *et al.* 2012). Information transmission depends on both the probability distribution of the input and the conditional probability distribution of the output for each possible input (Sarkar *et al.* 2020).

Signalling pathways are inherently very complex, with the transmission of signal at each step in a pathway requiring any number of protein-protein interactions, conformational changes, enzymatic reactions, degradation events; in many cases we may not have a complete understanding of all of the components involved. MI offers us an alternate approach (Brennan *et al.* 2012), considering a pathway or network to be a black box communication channel (Rhee *et al.* 2012), with only inputs and outputs (Tkačik *et al.* 2008*a*) (Fig. 2). Since calculation of MI requires response data from many hundreds or thousands of single cells, it follows that the method by which the response is measured in those cells must be taken into consideration, with any variability contributed by the experimental set-up minimised. High content imaging lends itself particularly well to the quantification of MI (Voliotis *et al.* 2014, Garner *et al.* 2016, 2017), making it possible to standardise image acquisition and analysis of an inordinate number of fixed or live cells, limited only by computing power and storage space.

The application of MI to a biological system was first carried out in a study of transcriptional regulation in *Drosophila* (Tkačik *et al.* 2008*a*,*b*), and later it was applied to signalling by the proinflammatory cytokine, tumor necrosis factor (TNF) (Cheong *et al.* 2011). One particular advantage of the calculation of MI is that it enables the comparison of multiple signalling pathways using the same metric (bits) (Sarkar *et al.* 2020). The number of bits as available input is logarithm to the base 2, such that eight distinct concentrations of hormone or cytokine gives a 3 bit input ($2^3 = 8$), but when Cheong and colleagues monitored the response of many individual cells to various concentrations of TNF through nuclear accumulation of either NF-κB (RELA) or ATF-2 transcription factors,
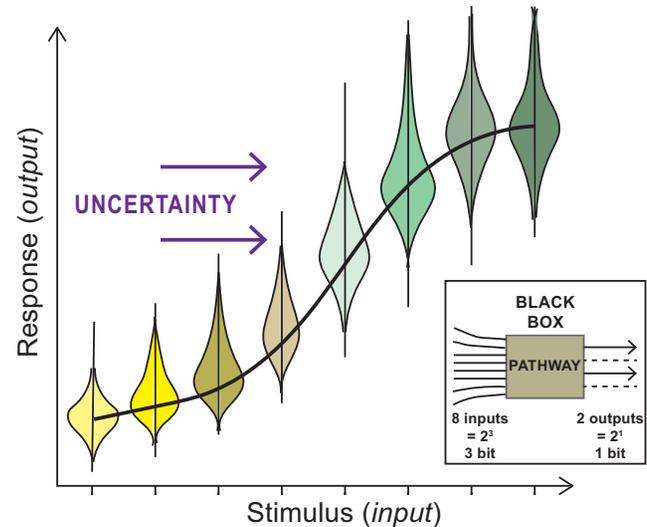

Figure 2

Information transfer along noisy communication pathways. MI can be used to assess how well an input distribution (range of stimulus values, doses of hormone, drugs) can be predicted from the range of output response obtained alone. Population average measures (strong black line, sigmoid curve) tend to obscure the inherent cell–cell variability in responses that exist within a population of cells, and due to this variation it can be difficult to accurately predict the stimulus range from the response (there is more uncertainty due to overlapping responses at a range of inputs). *Inset*: MI disregards signalling pathway components, but instead considers the pathway to be a black box. MI is measured in bits; eight concentrations of hormone or drug gives a 3 bit input. However, most studies yield an MI of ~1 bit (or less), indicating that each cell is only able to distinguish between two states of its environment (e.g. two concentrations of input). Some studies reveal an MI of 1–2 bits, however, indicating that two to four states of the environment might be distinguished between (dashed lines).

despite a 3 bit input, the calculated MI was only ~0.9 bits through each pathway. Sensing of both pathways simultaneously increased information transfer marginally to ~1 bit (Cheong *et al.* 2011), and indeed similar values of ~1 bit have been found for other signalling pathways, for growth factors, nerve growth factor (NGF) and pituitary adenylate cyclase-activating peptide (PACAP) (Uda *et al.* 2013), EGF (Voliotis *et al.* 2014), and in gonadotropin-releasing hormone (GnRH) signalling (Garner *et al.* 2016, 2017).

An MI of ~1 bit has been interpreted as an individual cell is only able to unambiguously distinguish between two states of its environment ($2^1 = 2$). Simply put, each new piece of information the cells receive enables them to distinguish between two different states, although how a cell responds to a signal will depend also on its prior understanding of its environment (Voliotis *et al.* 2014). MI is unable to define which are the two states that can be distinguished between, or to what degree (Jetka *et al.* 2019), however, but the more times the signal is sent

along a channel, the more sure the receiver can be about the nature of the input signal distribution (Voliotis *et al.* 2014). Despite this, an MI of 1 bit has led some researchers to consider a signalling pathway as producing a binary or switch-like response (Zhang *et al.* 2017), yet signalling pathways are dynamic in nature, and this interpretation conflicts with several studies that demonstrate a dose–response relationship between input and output to the TNF pathway (Tay *et al.* 2010, Lee *et al.* 2014, DeFelice *et al.* 2019).

Perhaps more conclusive or informative have been studies of how information transfer can be shaped and refined. As discussed previously, noise can restrict information flow through a channel, and in support of this Cheong and colleagues found that if they reduced their sample size to small fields of 14 neighbouring cells (likely recently derived from the same ancestor, and therefore phenotypically similar), the range of cell–cell variability was reduced, and MI increased to 1.8 bit, indicating that under these conditions the system could distinguish between two and four individual states (Cheong *et al.* 2011). Indeed, reducing the cell population in this way might be more reflective of sensing by an individual cell than assessing MI over larger cell numbers, since it is a single cell that has to sense its environment and make appropriate decisions. Selimkhanov and colleagues also demonstrated that reducing the noise made information transfer through a signalling pathway more efficient. This group monitored single live-cell signalling over time through mitogen-activated protein kinase 1 (MAPK1, ERK), calcium (Ca^{2+}), and nuclear factor kappa-B (NF-κB, RELA) and found that dynamic responses contain more information than static responses (Selimkhanov *et al.* 2014).

MI is a statistical measure of the amount of information transmitted along a channel, and the closely related metric, Channel Capacity (CC), gives a measure of the maximum number of stimuli a cell can distinguish (Rhee *et al.* 2012). Zhang and colleagues looked again at TNF signalling, this time in a panel of human cancer cell lines, and found that by excluding the subpopulation of non-responding cells, a CC value of >1.7 bits could be obtained, indicating that cells did have the capacity to distinguish between two or four states of their environment, and the researchers inferred that these cells are indeed capable of more than switch-like, or on-or-off-type responses (Zhang *et al.* 2017).

Negative feedback serves to attenuate or curb signalling – thereby fine-tuning or clarifying the signal – and this can be demonstrated elegantly at the single cell

level using MI. Cheong and colleagues found that A20-mediated negative feedback increased MI (although it remained ~1 bit) (Cheong *et al.* 2011). This observation was subsequently expanded upon in studies looking at different types of information transfer, with wet-lab experiments supported and enhanced by mathematical modelling. Voliotis and colleagues examined the effects of basal (leaky) network activity and cell-to-cell variability on information transfer in EGF signalling and found that in the presence of basal activity, ERK-mediated negative feedback (through phosphorylation of Raf-1 or SOS Ras/Rac guanine nucleotide exchange factor 1) increased MI by preventing the loss of dose–response and reducing the sensitivity to variation in substrate expression levels (Voliotis *et al.* 2014).

GnRH is central in the control of reproduction, promoting the synthesis and secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH) from gonadotropes in the anterior pituitary gland; like EGF, GnRH signals through ERK (but which it does so via phospholipase C-mediated activation of protein kinase C). We manipulated ERK-mediated negative feedback in this system to determine its effect on MI, denoting it 'fast' ERK-mediated negative feedback (5–15 min), and compared this with 'slow' negative feedback (beyond 40 min) via ERK-mediated upregulation of dual-specificity phosphatases (DUSPs), which requires time for transcription and translation (Garner *et al.* 2016).

To examine the effect of fast ERK-mediated negative feedback on information transfer through the GnRH pathway, we first knocked-down endogenous ERKs and expressed either 'wild-type' (WT) ERK2-GFP or catalytically inactive K52R ERK2-GFP using adenoviruses. Basal dual-phosphorylated (pp)ERK was increased when K52R ERK2-GFP was expressed, revealing the constitutive activation that is normally tempered by negative feedback. Notably, MI was decreased in the cells expressing K52R ERK2-GFP relative to WT ERK2-GFP, demonstrating that ERK catalytic activity (and therefore ERK-mediated negative feedback) improves GnRH sensing (Garner *et al.* 2016).

Likewise, we found that increasing slow negative feedback was also detrimental to GnRH sensing. To do this we used an ERK-responsive Egr1 promoter to express WT DUSP5, a nuclear phosphatase specific for ERK, or R53A/R54A DUSP5, a mutant unable to interact with and therefore unable to inhibit ERK. Expression of WT DUSP5 reduced ppERK, a suppression that was not seen for R53A/R54A DUSP5, and MI was also lower for the cells expressing WT DUSP5. Using stochastic modelling, we found that for both slow and fast negative feedback,

MI is maximal at intermediate levels of negative feedback (Garner *et al.* 2016).

A key feature of the GnRH system is that GnRH is a pulsatile hormone, with the maximal amounts of LH or FSH secreted at specific, sub-maximal pulse frequencies, so in our next study, we investigated whether more information could be gained by sensing during and after a second pulse (Garner *et al.* 2017). We developed a hybrid deterministic/stochastic mathematical model alongside our wet-lab experiments in which we were able to ask what effect cell–cell variability has on the ability of the system to sense GnRH through more than one pulse, and indeed this made it possible to deduce the state of variability present in our wet-lab system.

Introducing stochasticity into the concentrations of two effectors, the GnRH receptor (GnRHR) and calmodulin, we found that when the effector levels were rapidly fluctuating, additional information could be gained by sensing two pulses, but when the levels of effectors were largely unchanging (despite considerable cell–cell variability), the responses in the second pulse largely reflected those in the first, indicating that no additional information could be gained by sensing the second pulse. To determine how this model related to the situation in HeLa cells, we transduced cells with adenovirus to express GnRHR and NFAT-EFP and used live cell high content imaging to generate single cell traces of signalling from GnRH to NFAT. We found that our experiments closely paralleled a situation in which the levels of effectors were relatively stable, and little additional information could be gained through sensing a second GnRH pulse (Garner *et al.* 2017).

An important point to take away from studies of information transfer goes against our traditional understanding of signalling pathways. We learn that a signalling pathway amplifies a signal, as each receptor activates multiple effectors, and this is increased at each step of the pathway. But indeed, information theory tells us instead that information cannot be created, only lost due to noisy processing (Tkačik *et al.* 2008*a*). Any increase in signal amplitude from one tier to the next in the cascade must be coupled to an increase in cell–cell variation (Voliotis *et al.* 2018), so the question becomes, how do cells mitigate this loss of information (Garner *et al.* 2016)?

Significance

Variation between cells can be crucial during differentiation and development for the establishment of initial asymmetries that are reinforced to determine cell fates (Elowitz *et al.* 2002), but cellular heterogeneity is also essential to enable cell populations to adapt to changes in their environment (Kaern *et al.* 2005). We can sense this in our cell cultures when cell lines are put under stresses of irregular feeding and splitting schedules or a change in media or even a failing incubator.

If cells in a clonal cell population show distinct cell–cell variability, it is reasonable also to assume that cells in tissues in vivo show the same variability, likely more pronounced. And since cells in a clonal cell population exhibit variability in their response to hormones, cytokines, growth factors, and drugs that we administer in vitro, it follows too that cells in our body, perhaps in diseased tissues, will have a varied response to drug treatments.

In cancer, signalling pathways that wouldn't usually be used are upregulated, making cancer cells different from normal body cells, and it is these differences, through differences in gene expression, that enables cancer to undergo uncontrolled proliferation and evade the immune response. These aberrant pathways are often targeted in cancer therapy (often known as targeted therapy), yet many patients eventually develop resistance to these drugs. It is now known that tumour cell heterogeneity can play a key role in this; by killing the drug-sensitive cancer cells (termed 'fractional killing' (Berenbaum 1972, Spencer *et al.* 2009)), this can enable the unchecked proliferation of drug-insensitive cells, which eventually dominate the tumour (Kim *et al.* 2018).

In light of this, Cohen and colleagues used time lapse automated fluorescence microscopy to track how ~1000 proteins in individual human H1299 lung carcinoma cells responded to camptothecin, a drug used in the treatment of cancer that targets topoisomerase-1 to cause cell death (Cohen *et al.* 2008). Before the drug was administered, all of the proteins assessed elicited cell–cell variability in their expression levels, only 20% of which could be attributed to variance in cell cycle stage. Cell–cell variability increased over time by ~30% on average after a day. Groups of functionally related proteins exhibited similar dynamics and protein localisation profiles, but a subset of proteins were found to be very different in their responses. At least two of these proteins showed cell–cell variations that correlated with the fate of the cells, and these findings were able to give clues about how a subpopulation of cells are able to escape the drug action.

Although the removal of a subset of cells is detrimental in the treatment of cancer, the selective removal of a

fraction of cells in the treatment of age-related and chronic diseases has been shown to be beneficial (Song *et al.* 2020), and high content imaging is also being used to identify and track these cells (De Cecco *et al.* 2011, Udono *et al.* 2012).

Conclusions

High content imaging is a high-throughput, fluorescence microscopy method that can be fully automated to enable unbiased and standardised quantification of signalling dynamics in single cells. Along with single cell 'omics technologies, studies using high content imaging are revealing how variable individual cells are in their expression of cell components and in their response to stimuli. Single cell data can be used to quantify information transfer (mutual information, MI) through intracellular signalling pathways, taking account of the noise. Recently, Jetka and colleagues released an R package that will enable computational biologists with only limited understanding of information theory to perform MI and CC analyses on their single cell data (Jetka *et al.* 2019). In the treatment of disease, cellular heterogeneity in cancer is key in the development of drug resistance, where targeted therapies eliminate drug-sensitive cancer cells, allowing drug-insensitive cancer cell population to expand unchecked. In contrast, the removal of a subset of cells has been shown to be beneficial in the treatment of age-related and chronic diseases.

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