Systems Biology in Single Cells

Macdara Glynn, Damien King and Jens Ducrée

Abstract From the beginning of the twenty-first century, there has been a shift towards studying biological processes using a holistic rather than a reductionist scientific paradigm thus establishing the approach now named "systems biology" or "systomics". This method of biological investigation represents a synergy where life sciences, systems engineering, and information technology examine the interactions between biological pathways, rather than solely focusing on individual pathways in an isolated manner. To date, systems biology has often studied population averages rather than individual characteristics of cells which might display a significant spread. However, as a single cell is the smallest operational biological unit that encompasses all metabolites necessary for maintaining a viable living entity, the application of systems biology approaches to the study of distinct cells is fast becoming a goal of many research groups. In this chapter we will describe some of the technologies that enable the isolation of individual cells in a form that accommodates systomics studies, the biological methods that are then deployed on such isolated cells to generate system-level information, and finally describe some of the bioinformatics that is specifically directed towards single-cell studies.

Keywords Systems level biology · Microfluidic lab-on-a-chip · Single cell analysis · Integrated biology · Single cell optical detection

1 What Is Systems Biology?

As it is the case with many emergent scientific disciplines, in its early days a generally agreed definition or understanding of the goals and boundaries of systems biology was rather elusive. Westerhoff and Alberghina [1] compare this poor definition of identity to that experienced by molecular biology in its embryonic

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period where it was considered a nexus of crystallography, theoretical biology and chemistry. However, systems biology has, in the last number of years, come to represent a scientific paradigm where emergent properties and functionalities of living entities are examined from a perspective that encompasses a repertoire of often disparate biological pathways (Fig. 1). The interplay between these pathways is studied using high throughput and quantitative experimental strategies that allow the biological system to operate in its intact state, and data is then used to generate predictive mathematical models that can be queried to pose new biological questions. Crucially, systems biology represents a field where multiple disciplines including biochemistry, information technology, molecular biology, mathematics, physics and systems engineering must operate in close harmony in order to address the questions posed. Although all these disciplines have been around for some decades, their integration towards a common endpoint is relatively new [1].

Due to its ability to design, record and model vast amounts of data, many investigations of systems biology operate at the large-scale "-omics" level—i.e. genomics, proteomics and metabolomics [3]. This helps to recognise emergent phenotypes that may otherwise be too subtle to be identified without combining the big and quantitative data sets generated, with the mathematical modelling capabilities used in these studies [2]. Combining these individual "-omics" through systems biology can then lead to a higher level modelling of the "interactome".

1.1 Systems Biology at the Single Cell Level

Many studies (at both systems level, and at the classical, single-pathway level) have produced their data based on signals obtained from a large cohort of cells—termed either "population-level studies" or "bulk assays". However, such multi-cellular studies may not always identify minute changes that take place at the single cell level, particularly when the fate of a single cell within a population represents a low-frequency occurrence that results in the single cell presenting a phenotype distinct from the population (Fig. 2a). When measured at the population level, rare single-cell phenotypes can be lost to background noise in the data. This is shown by Tay et al. where they demonstrated the activation of cellular pathways in response to tumour-necrosis factor (TNF-α) over a concentration range covering four orders of magnitude.

By examining a large number of cells at the single-cell level using a systems biology approach, the heterogeneous nature of TNF-activation across the population was examined. It was found that cells may activate TNF-αdigitally, and critically, a small subset of cells were shown to activate on a lower dose exposure [4]—this was in contrast to population-based studies [5]. Alternatively, in cases where a treatment will generate more frequent but polarising phenotypes, a population-level study may suggest that the response is lower but distributed in all cells, rather than showing that distinct subpopulations emerge (Fig. 2b). For example, the response of the well-studied "defender of the genome" protein, p53, to irradiation occurs strongly in

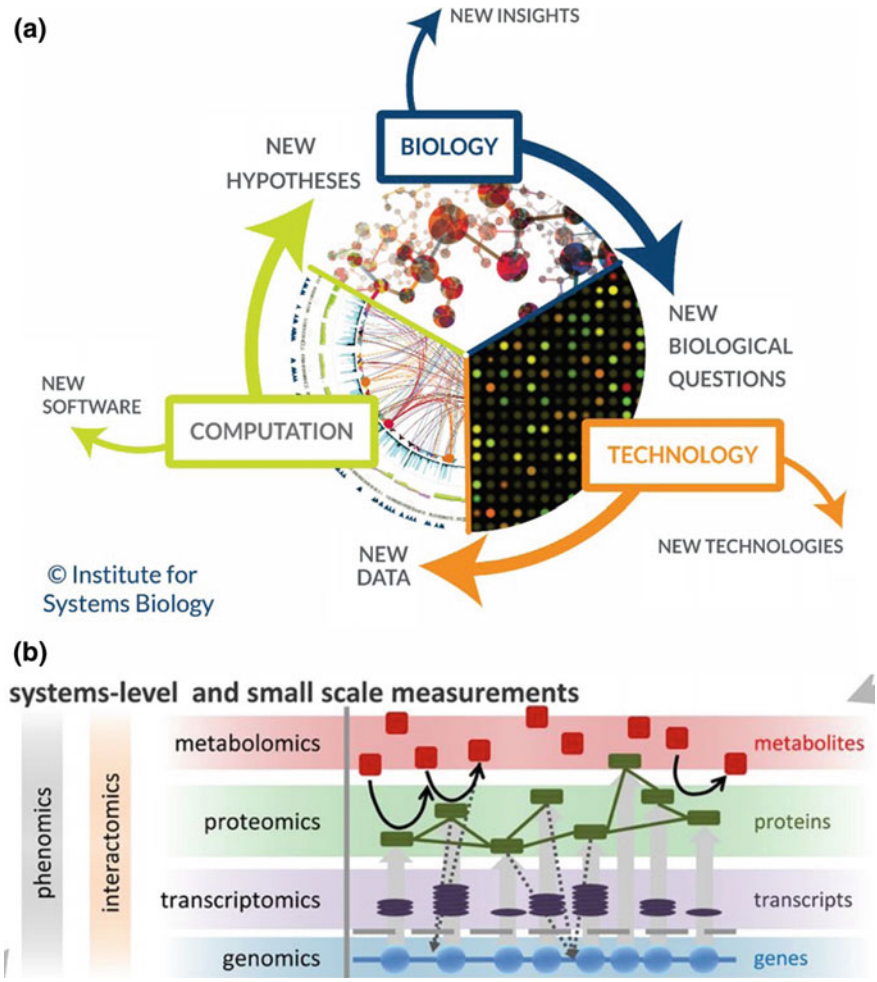


Fig. 1 Overview of the multidisciplinary and integrative nature of systems biology. **a** The innovation engine as demonstrated by the Institute for Systems Biology. **b** A visualisation of the interactions between various—omics as shown by Mast et al. [2]. Both show multi-aspect biological experiments are designed and executed using biochemical, molecular and cellular techniques. High throughput and multi-parameter technological systems (often optical-based) monitor the progress of the experiment in real-time and record data. These large and multi-dimensional datasets are mathematically analysed to identify dependencies and interactions between the biological parameters examined, and models are generated. These models are then refined and adapted as new biological questions are identified and experimentally examined. (**a**) Reprinted with permission from the Institute for Systems Biology. (**b**) Reprinted with permission from Ref. [2]. Copyright 2014 The Rockefeller University Press

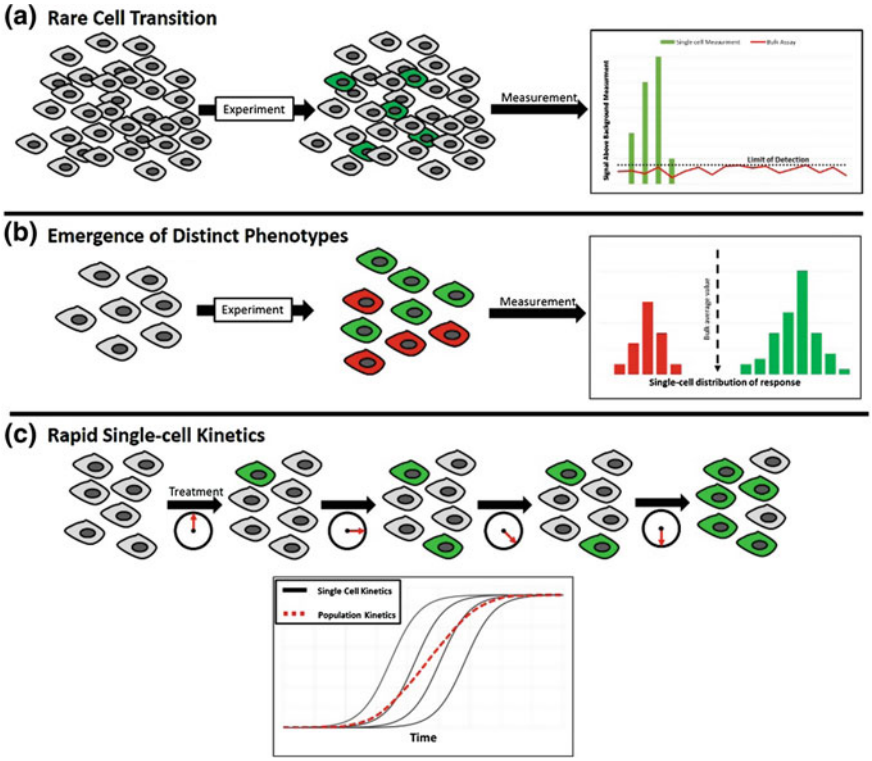


Fig. 2 Population-based bulk systems biology versus single-cell systems biology. **a** Rare cellular transitions in a large background will often not generate sufficient signal above the bulk background to be quantifiable, or even detectable. When each cell is measured individually and compared to the background, the same experiment can identify a small number of cells undergoing transition. **b** In cases were cells can transition to one of a number of phenotypes, single-cell studies can identify such bimodal distributions. **c** Rapid cellular transitions that are slow to spread through a population can be misinterpreted when examined at a population level

a sub-group of treated cells, but, if at all, only at a low level (if at all) in the other cells [6]. If examined at a population level, this dual-phenotype is not observed, and it rather appears that all cells equally respond at a lower intensity.

As a final example of the benefit of single-cell studies over population studies in systems biology, one may examine the effect of rapid, time-dependent phenotypic transitions (or gene activations) that occur at the single cell level but spread in time through the population (Fig. 2c). When examined individually, it is clear in these experiments that, when activated, the transition is rapid on a per-cell level. However, if one were to observe the bulk transition at the population level (by, for example, monitoring the production of an associated soluble and detectable factor in the culture media), it would appear that the overall transition kinetics were slow and gradual, rather than rapid but digital at the cellular level [7].

In this chapter, a number of technologies will be presented that enable systems biology to be studied on cell-by-cell basis. The chapter will be sectioned into methods of isolating or tracking single cells, the strategies of biological systems experiments used on isolated cells, and the methods of high-throughput optical monitoring of these experiments. Although these headings are used for the sake of convenience, it should be remembered that amalgamation of all these disparate disciplines into a single workflow is required to fully leverage the analytical power of systems biology.

2 Standing Out from the Crowd—Working with Single Cells

The resolution of data that can be generated on a single-cell level in a systems biology study is limited (to a first approximation) by the reliability and consistency of the instrumentation. Unlike many other single-cell level methods (such as fixed-cell immunofluorescence), the technologies used in systems biology often require cells to be maintained as viable and actively metabolising, thus allowing interaction between events in seemingly distinct and those in other cellular pathways in response to experimental conditions. Furthermore, these technologies will need to present single cells in a framework where high-speed and multi-channel optical and chemical detectors can maintain contact and record data in real-time.

Broadly speaking, there are three approaches ensuring this spatio-temporal resolution of data. Each displays specific advantages depending on the sensitivity of the cell of interest to its environment, or depending on the cellular output under investigation. In the first approach, cells can be maintained in co-culture within a population, but optical systems are employed to target a particular cell of interest and track the cell throughout the length of the experiment. This is useful, for example, when a cell is sensitive to low population density in its neighbourhood and can be lost through a process called anoikis—a type apoptotic response to the absence of cell–matrix interactions. In such cases, tracking the cell while it is maintained in a population is favourable.

In the second approach, technology is employed to physically capture or otherwise select and segregate single cells from a wider population where they can be treated and monitored, often with high levels of minute control over the surrounding environment. For example, when the secretion of soluble constituents from a single cell represents an impact factor, this approach allows the cell to be confined to a defined environment where a change in the local concentration of the soluble factor can be directly attributed to the contained cell.

The third approach is related to the second and involves processing a large population of cells through a multi-optic detection locus. Critically, even though the input sample is a bulk population, the resolution occurs at a cell-by-cell level. Flow cytometers and cell sorting devices are commonly used for such studies. However,

instruments such as the Amnis ImageStream [8] has integrated the single cell and multi-optical capabilities of a flow cytometer with a high-speed fluorescent microscope to provide a large amount of fluorescent and molecular localisation data at single-cell resolution. Although high depth cellular data can be produced and examined using a systems biology approach, each cell is only examined at a given, potentially random point in time as it flows through the detection locus; hence, kinetic data and time-dependent dynamic interactions within a specific cell are lost.

2.1 *Tracking and Monitoring Selected Cells in a Wider Population*

Microscopic monitoring of individual cells within a population throughout the duration of an entire experiment (generally also requiring that multi-channel optical systems can record data simultaneously) remains challenging. Only a few microscope setups offer a combination of high-resolution micro-displacement stages with controlling software that can recognise and track mobile targeted cells (often undergoing significant morphological change). These instruments have greatly enhanced the ability of systems biology to operate at the single-cell level. Long-term monitoring of single cell experiments—often continuing for more than 24 h—commonly requires vibration-free conditions for the setup.

There are a number of proprietary software packages that implement cell tracking which share a similar strategy. First, the operator places a region-of-interest (ROI) around the target cell. An algorithm then determines the centre of the cell, and establishes a set of features for tracking the translocation of the centre of a cell and its morphological change along subsequent frames. Upon larger movement beyond the starting position, the micron-scale resolution stage is repositioned to home on the new location of the cell. As long as the stage facilitates micron-level adjustments, a cell can be reliably tracked. In addition to such X-Y stage displacement, most modern systems also allow fine tuning of the Z-axis for investigating cells suspended above the focal plane. Sub-micron adjustment of the Z-axis even allows the selection of vertical optical slices, thus enabling spatio-temporal 4D recording of the cells for rendering and modelling (Fig. 3).

Although more challenging, such 4D tracking has revealed dynamic intra-cellular interactions leading to unexpected cellular motions. For example, using such a microscopic system, Kaczmarczyk et al. demonstrated that, when expression of the centromeric protein CENP-W is knocked down, consequent effects on the mitotic spindle brings about a rolling motion in cells that are attempting to undergo mitosis [9]. Using a similar system, but following a strategy where single cells were monitored after an on-instrument, time-controlled chemical treatment, Yivgi-Ohana et al. developed a novel tool to study progression of the highly complex apoptotic cellular system in real-time in living cells [10].

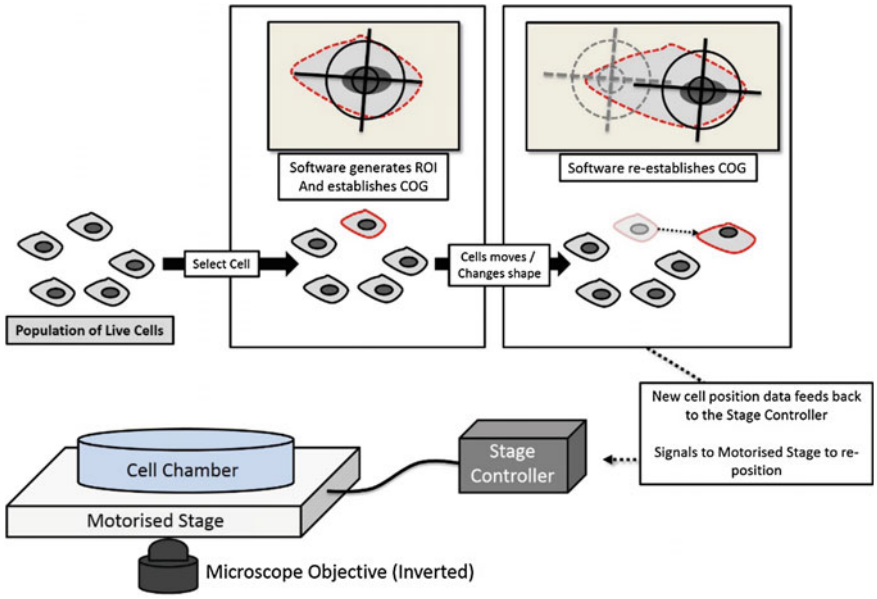


Fig. 3 Tracking of individual cells from a population of live culturing cells using a live-cell imaging microscopic platform with a high-resolution micro-displacement motorised stage

2.2 Segregating and Isolation of Single Cells from a Population

For cells that are stable when examined in isolation, it can be confined to a geometrically and chemically well-defined environment. This way the operator can re-visit the same cell repeatedly and ensure (with suitable in situ sensors) that measured changes to the environment are due specifically to the presence of the captured cell. Furthermore, the interdisciplinary collaboration between biology, microfluidics and microfabrication has leveraged high-throughput interrogation of individual cells. This is a significant advancement from classical, laborious methods such as micro-manipulation where individual cells had to be identified and hand-picked by an operator. Microfluidic single-cell isolation systems use a number of distinct strategies, some of which will be described here.

As the depth of microfluidic systems can be precisely chosen to avoid vertical stacking of cells, fine z-axis control of associated optical or other detection hardware is not as critical as it is with 4D microscopy. Moreover, strict laminarity of flow is imposed by the micro-confinement, thus offering well-defined, convection-diffusion conditions for imposing spatio-temporally stable concentration profiles and shear rates. This allows a high level of physic–chemical control and reagent exposure [11]. In free-flow microfluidic convection-diffusion systems featuring with interconnected channels and chamber [12], physic–chemical gradients

can even be generated, e.g. to establish different concentrations of a chemical stimulus at a subcellular resolution where either side of a single cell is exposed to a distinct chemistry [13]; or a temperature gradient applies between across an embryo [14]. In both these examples, the microfluidic system also permitted real-time optical monitoring of the living cells using fluorescent markers. Other microfluidic strategies enabled the generation of concentration gradients that could be applied to microfluidic test chambers in order to facilitate the effect on individual cells. These concentration profiles were imposed using a set of inputs where chemicals of a defined concentration. The chemicals were pumped through a network of interconnected serpentine channels that merged at the experimental chamber to expose cells to a concentration gradient and allowed chemotaxis of individual cells within this gradient to be monitored optically [12].

In addition to subcellular resolution, the capacity of microfluidic systems to integrate a comprehensive array of distinct test environments to a single chip permits high throughput and high fidelity systems-biology experiments. Many more recent microfluidic platforms isolated and examined a large number of individual cells on a small-footprint "chip" that could be manufactured in a cost-efficient fashion, either in bulk by a manufacturer or even made to order in the laboratory of the research group. Researchers have used a diverse assortment of microfluidic architectures to allow such high-throughput single-cell isolation, ranging from passive filtering to active bio-physical methods, some of which will be described below.

Various microfluidic chip technologies provide methods for individual cell capture, as well as rapid exchange of reagents and optical observation of the response of the captured cells to these reagents over time. Single cells can be arrayed by passage of a population through a series of physical barriers with geometries that can only retain a single cell. Rowat et al. developed a syringe-driven microfluidic chip comprised of a number of yeast capture channels in which single cells from a population can resolve. This occurs as a cell travels to the end of the chamber where it is plugs into a fluidic constriction. The resulting increase of the flow resistance to divert subsequent cells to other trapping sites increases the probability of subsequent cells of entering a bypass channel, thus making it very likely that a single cell occupies the capture channel.

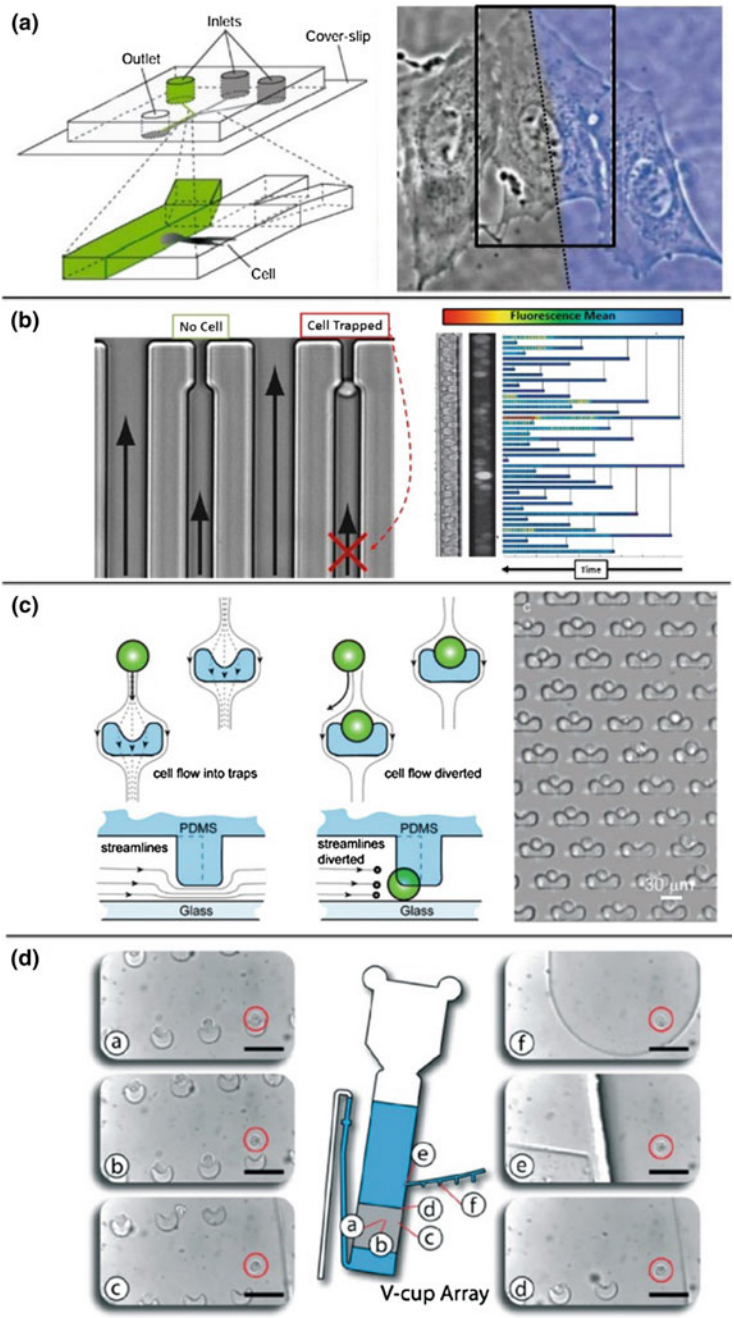
Using in situ fluorescent microscopy on live cells, the researchers were then able to follow multiple phenotypic variations in the progeny of the captured cells as these were constrained to grow in a line along the chamber [15]. Wu et al. elegantly produced a microfluidic chip with the capability of trapping single cells in a 70-pl enclosure using actuated soft-polymer valves to close off a microfluidic channel, thus generating the reaction chamber. The actuation of the valves is reversible and individually controlled, and so allows random exchange of media and reagents by the operator [16]. As the chamber is hydrodynamically sealed, this device can monitor the media surrounding the captured cell for the efflux of metabolic targets in real time; Cai et al. used this functionality to monitor the protein expression in yeast cells at the single molecule level in real-time [17].

Another microfluidic architecture designed to isolate single cells for systems level investigations uses post-like barriers specifically shaped to accommodate a maximum of one cell as a population of cells travel through the micro channel. For efficient capture, the number of barriers exceeds the expected count of incoming cells. This ensures that there is a barrier available for each cell of interest. As the population progresses through the array, they occupy the available capture loci, but if a cell encounters an occupied barrier the structure is designed such that it will continue travelling until an empty post is encountered. When the population is fully resolved, cells are arrayed at well-defined positions throughout an experiment, even under changing physic–chemical conditions.

DiCarlo et al. built such a single cell isolating structure using flow through arrayed suspended obstacles [7]. Cells and chemical components were introduced to the array using a syringe and the overall structure used branched delivery channels to ensure a high uniformity of flow across the chip. Streamlines carry cells to the capture obstacles which consist of two layers of cup-shaped trapping sites—40 and 2 μm. The larger gap serves as the primary fluidic conduit to carry cells to the trap, and once resolved to the trap, a cell blocks the smaller, 2-μm gap to thus throttle the flow through this constriction. As a result, subsequent cells are diverted towards empty traps located further downstream to eventually induce a single-cell occupancy distribution. By incorporating the chip onto a fluorescent microscope, the group monitored individual membrane-bound and cytosolic carboxylesterase concentrations in live cells in a number of cell lines at single cell resolution, and also examined inhibition of these enzymes using NDGA [18].

Burger et al. further developed the post-like barrier architecture by integrating them onto a rotating chip. This "lab-on-a-disc" operates in a hydrostatic, "stopped-flow" regime to eliminate the need for self-sealing valves to re-direct flow when a cell occupies a trap. Under rotationally induced artificial gravity, cells sediment along straight radial lines until trapped by an empty obstacle termed "V-cup" in their path. Such an innovation notably increases the number of traps with single-cell occupancy. Interestingly, the group also combined the microfluidic chip with a customised "optical tweezers" module. Following distribution and live-cell fluorescent analysis of a cell population, the group could select individual cells of interest from the array and relocate them to a separate reaction chamber on the chip. In the future, such array-based technology may enable systems level translational biology where, for instance, leukocytes from a patient sample can be pre-selected and screened based on membrane markers and fluorescent imaging; from here rare cells can be further individually isolated and removed from the array for subsequent testing [19].

Microfluidic optical tweezers were also combined with microfluidic chips by Eriksson et al. for single cell live-cell systems monitoring of the cycling of fluorescently tagged proteins (Mig1 and MSN2) between the cytosol and nucleus in *S. cerevisiae* cells as environmental glucose levels were adjusted. Here, the tweezers arrayed individual cells in a spatial pattern within a rapidly adjustable chemical gradient. The cycling kinetics of proteins of interest was then monitored in real-time as the gradient was manipulated [20].


(a)
Inlets
Cover-slip
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(b)
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Glass
30 μm
(d)
a
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V-cup Array

◀ **Fig. 4** Examples of microfluidic techniques and strategies used for either segregation of individual cells from a population or chemical treatment of cells at resolutions applicable to systems-level single-cell studies. **a** Demonstration of the minute control of chemical diffusion achieved by Takayama et al. allowing barrier-free chemical gradients to be generated at a subcellular resolution where either side of a single cell is exposed to a different chemistry. The microfluidic architecture is shown on the *left* and an image of the fluidic barrier (*hatched line*) over a single bovine capillary endothelial cell (outlined with a *black box*) is shown on the *right*. **b** Single-cell yeast capture as performed by Rowat et al. in which fluidic streamlines carry a cell to the end of a channel where it blocks an outlet and minimises the probability of further cells entering the channel (*left*). Progeny of the captured cell are confined such that each can be individually monitored in real-time for metabolic events as changes to the environment are induced (*right*) [15]. **c** Flow through arrayed suspended obstacles by DiCarlo et al. designed to direct cells to traps where they (similar to b) block a fluidic outlet thus minimising the chance of further cells resolving to an occupied trap. While in the traps, surrounding chemistry can easily be adjusted and live imaging of the cells used to monitor systems level outputs. **d** An adaptation of arrayed obstacles by Burger et al. but carried out on a centrifugal chip under stagnant-flow conditions without the need to control and valve fluidic streamlines. Circled a-f labels indicate the journey of a selected individual cell as it is picked up from its capture trap and relocated to a reaction chamber on the chip for further analysis. (**a**) Reprinted by permission from Macmillan Publishers Ltd: Ref. [13], Copyright 2001. (**b**) Reprinted with permission from Ref. [15], Copyright 2009 National Academy of Sciences, USA. (**c**) Reprinted with permission from DiCarlo et al. Ref. [7]. Copyright 2006 American Chemical Society. (**d**) Reprinted with permission from Ref. [19] Copyright 2015 Royal Society of Chemistry

Another strategy for single cell isolation and reaction monitoring in high throughput involves the use of droplet microfluidics. Here, cells are suspended in an aqueous solution or medium which is then suspended in an immiscible hydrophobic carrier liquid (generally a silicone-based oil). This way the droplets act like reaction chambers to define the physic-chemical microenvironment of each cell. The droplet can then be shuttled and directed along pathways as required using pressure fields imposed by pumps and flows through intersecting channels. Importantly, this architecture allows further chemistries or reagents to be specifically delivered to individual droplets by merging with additional droplets carrying the desired reagent.

Many systems have been developed in which droplet-based microfluidics has leveraged continuous and extremely high-throughput experimentation. Konry et al. used droplet microfluidics to monitor micro-size remodelling of the cytoskeleton and microtubule polymerization in dendritic cells at the immunological synapse formed between co-encapsulated pairs of live dendritic cells and CD4 + T cells in droplets [21]. Other groups have also integrated high-throughput droplet sorting on chip-based systems, e.g. to remove empty droplets or to select droplets containing cells of interest based on the presence of a particular fluorescent marker [22]. These strategies are shown in Fig. 4.

3 Biological Systems Used for Single Cell Systems Biology

In addition to the selection of an appropriate method of identifying and/or isolating single cells for use in systems level studies, the types of biological assays and chemistries that are to be incorporated to the study need to be taken into account to avoid compromising the quality of data. Non systems-level experiments may be considered to offer more freedom to apply well established bioassays to cell studies as these often investigate pathways at fixed points in time or even in fixed cells that are no longer alive and thus ceased to metabolise. For real-time, multi-parameter systems investigations, particularly in single cells, assays must be selected where the chemistry used to adjust or monitor the experimental environment must not interfere with the continuous output generated by the experimental cell in an uncontrollable way.

A number of strategies exist for such studies, and are frequently designed to allow a molecular switch within a cell to be activated at a specific experimental point in time. This can then coincide with the activation of multi-channel, real-time monitoring of diverse cellular events such as dynamic expression of genes of interest, physical re-structuring or movements of the cell, bio-physical reactions of the cell, or secretion of molecules into the surrounding environment—to name but a few. These experimental designs can become quite complex and can require highly multidisciplinary collaboration. For example, stable transfection and cloning of new cell lines may be required to enable the cells to switch characteristic molecular pathways in a time-dependent manner, while high-resolution real-time microscopy is then required to follow the multi-point data emerging from these cells over time. When developing novel experiments and platforms, partnering with researchers carrying competence with the design engineering of microfluidic architectures (or other methods of single-cell resolution data acquisition) is required to tailor the test-platform to the targeted outputs of the research.

3.1 Quantitatively Monitoring the Fate of Single Cells Through Time: Example of Protein-based Systems Experimentation

Classical protein investigations in bulk and/or fixed time-point studies often involve the labelling of target proteins with an antibody carrying a fluorescent marker, or alternatively by using cells genetically modified to express a fusion version of the protein of interest with green fluorescent protein (GFP) (or one of its derivatives) expressed at the N- or C-terminal. These events are then either imaged at a given point in time (for example in chemically fixed cells), or measured in the bulk sample. Yet, in systems-level studies, these techniques are less powerful as phenotypical changes in response to the protein of interest are difficult to elucidate as the entire pool of the protein is constantly labelled. Recently, a novel type of

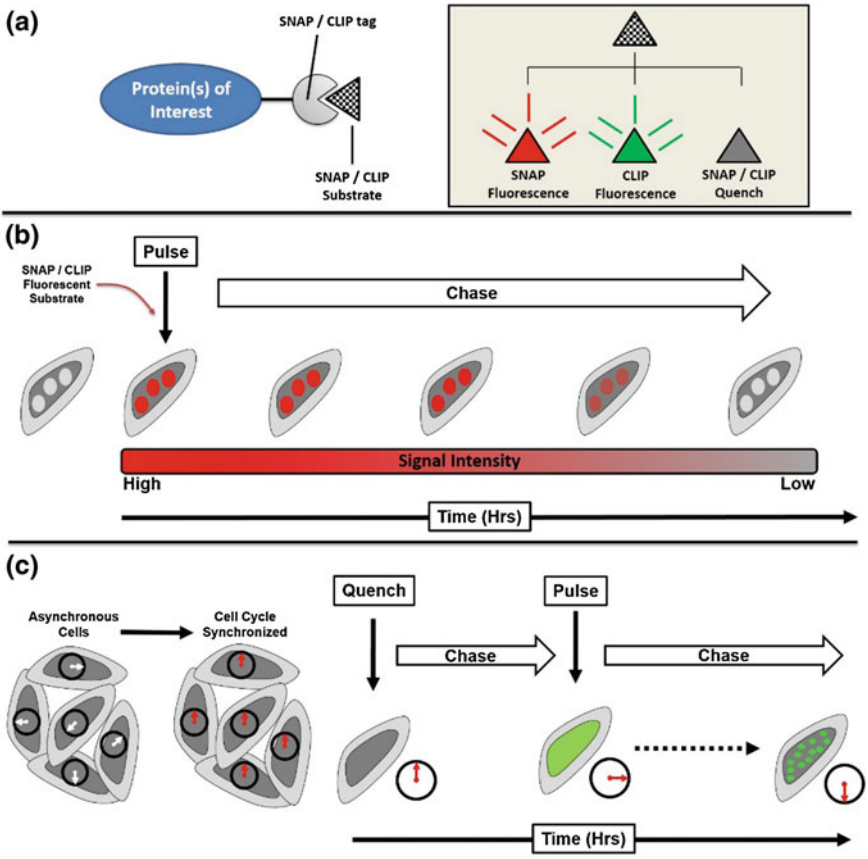

Fig. 5 The use of SNAP /CLIP tagging strategy to follow the dynamic fate of proteins in a single cell over long time scales. **a** SNAP/CLIP tagged protein of interest represented schematically. The various forms of the binding substrate are shown in the *greyed box*. **b** Experimental representation of a Pulse-Chase single cell investigation. Here, a protein of interest is monitored as the pool of protein in the cells at a point in time is fluorescently labelled and the diminishing fluorescence is measured over time in a single cell (to investigate protein turnover) or across cell generations (to investigate physical inheritability of the protein). **c** Experimental representation of a Quench-Chase-Pulse single cell investigation. Cell cycles in a population are synchronised (or the position in the cell cycle is otherwise measured for single cells) and the current pool of the protein of interest is quenched. The protein expressed in a selected window is fluorescently labelled, and the fate of this pool is monitored in time

molecular tag has been developed that can be biologically cloned onto a protein of interest and expressed (either stably or transiently) in a cell line. This tag (called SNAP-tag) provides a powerful tool for monitoring and tracking protein dynamics and half-life within living cells over longer intervals.

SNAP (Fig. 5a) is a genetically encoded protein derived from the human DNA repair enzyme O6-alkylguanine-DNA alkyl transferase (hAGT), and acts as a

suicide enzyme protein fusion tag that catalyses its own covalent binding to benzylguanine (BG), and its fluorescent derivatives [23]. Unlike most GFP-based fluorescent proteins; SNAP is therefore not, in itself, fluorescent but it can bind, under given experimental conditions, to a number of cell-permeable chemical derivatives that provide signals that can be followed in real time. More recently, a second tag similar to SNAP which uses distinct binding chemistry has become available, called CLIP-tag. Employing SNAP and CLIP in conjunction has allowed simultaneous labelling of two proteins of interest in the same cell, and concurrently follow the dynamic fates of them in real-time. There are two primary modes of use for SNAP/CLIP that are used in single cell systems level studies—Pulse-Chase labelling (Fig. 5b), and Quench-Chase-Pulse labelling (Fig. 5c).

In SNAP, Pulse-Chase labelling of live cells, protein(s) of interest are expressed in the cell with the SNAP tag expressed. At a specific time in the experiment, a fluorescent substrate is added to the cell medium that will bind the current pool of SNAP-tagged protein in the cell, thus rendering them detectable by real-time fluorescent microscopy. When the cells are exposed to the substrate for a defined, short interval, only the pool of protein that was present during exposure will be labelled, while fluorescence is absent on new protein that is expressed after removal of the substrate. This way changes in location, kinetics and turnover of this labelled pool of protein can be recorded without interference from any newly expressed protein expressed at a later stage.

As well as monitoring the turnover of a specific pool of a protein of interest in a cell, SNAP Pulse-Chase also allows tracking of the labelled pool of a given protein of interest to be followed through cellular generations, either by real-time monitoring in long-term experiments, or at fixed time-points in the pulsed cell and in its progeny. The SNAP tag method has proven invaluable in studying the highly complex biochemical system found at chromosomal centromeres. Using SNAP/CLIP Pulse-Chase strategies, Prendergast et al. [24]. and Bodor et al. [25] demonstrated the dynamic nature of the multi-faceted mechanism of centromere inheritance by characterising a number of the centromeric proteins as either physically inherited by daughter cells (such as CENP-A/Histone H.3), or non-inherited (such as CENP-T and CENP-W).

By additional use of an irreversible SNAP/CLIP substrate that is non-fluorescent, a Quench-Chase-Pulse labelling strategy can also be utilised. This allows serial labelling of SNAP-tagged proteins of interest, thus resolving when pools of a protein were expressed in a cell. At the beginning of an experiment, the cell is transiently exposed to the non-fluorescent substrate, known as the quench. This prevents the detection of the current pool of SNAP-protein in the cell. Following an experimentally selected interval (chase), the cells are then transiently exposed to the fluorescent substrate which will label only the protein pool that has been expressed since the end of the quench. When researchers couple this technique with the examination of individual cells in which the phase in the cell cycle is known, or the cell(s) have been synchronised prior to the quench, the timing of protein activity (loading, foci formation, co-localisation with other proteins) within a cell can be elucidated with great precision.

3.2 *Quantitatively Monitoring the Fate of Single Cells: Example of mRNA-Based Systems Experimentation*

The detection and monitoring of specific mRNAs in individual cells proves to be challenging, even if performed at a single target level. One of the more common methods of such investigations is the modification or adaption of an mRNA binding protein to generate a fluorescent signal upon binding to the specific mRNA of interest, thus allowing localisation of the mRNA species. However, this method is limited by the specificity of the binding protein for the targeted mRNA, often leading to inaccurate results. This can be overcome by a so-called MS2 system by modifying the gene for the mRNA of interest to co-transcribe an additional sequence known as the MS2 binding sequence (MBS). When an RNA binding protein (MCP) that specifically targets this sequence is modified to carry a fluorescent signal, the mRNA of interest can be measured in real time.

However, due to limitations arising from the number of fluorescent versions of the MCP protein, simultaneous monitoring is restricted to 3–4 mRNAs in a single cell, thus precluding the systems researcher to perform a comprehensive analysis of a large family of mRNAs simultaneously in a single cell. Another mRNA tracking method uses fluorescent in situ hybridisation (FISH) whereby an ssDNA probe with complimentary sequence can fluorescently label the mRNA of interest. The specificity of FISH probes to targeted mRNA is high, but generally restricted to two colours. Therefore, FISH is less amenable to multi-parameter systems biology that would require a larger number of mRNAs to be monitored simultaneously.

Recent developments by Lubeck et al. [26] have modified the FISH method to allow monitoring of up to 35 mRNAs. As opposed to the MS2 system, the cells must be fixed. However, the number of mRNAs that can be visualised simultaneously provides an experimental paradigm where multiple time-points are isolated and processed to allow the construction of a systems level kinetic model of gene expression. The method developed is an exemplary case for the interdisciplinary collaboration as key prerequisite of systems biology. The strategy necessitates application of technologically advanced Super-Resolution Microscopy (SRM) which can measure optical events emitted from features as small as 10–20 nm, and a number of photo-switchable fluorophore pairs that can be detected using SRM. Rather than using a single colour throughout the length of the ssDNA probe, barcodes based on three out of seven available colours are positioned along the probe at a resolution detectable by SRM. This allows biological experimentation in which a large number of specifically identifiable probes can be multiplexed in the same cell to follow the expression of a collection of genes related to a particular biological question through FISH hybridisation. Finally, due to the large amount of 3-dimensional (or 4-dimensional if time was included) data that must be processed to generate a model of the spatial arrangement of the targeted genes in the cell, an imaging method called stochastic optical reconstruction microscopy (STORM) is employed to analyse the data. As a proof of principle, the strategy profiled 32 mRNAs involved in the stress response of 60 individual *S. Cerevisiae* cells when exposed to stress-inducing

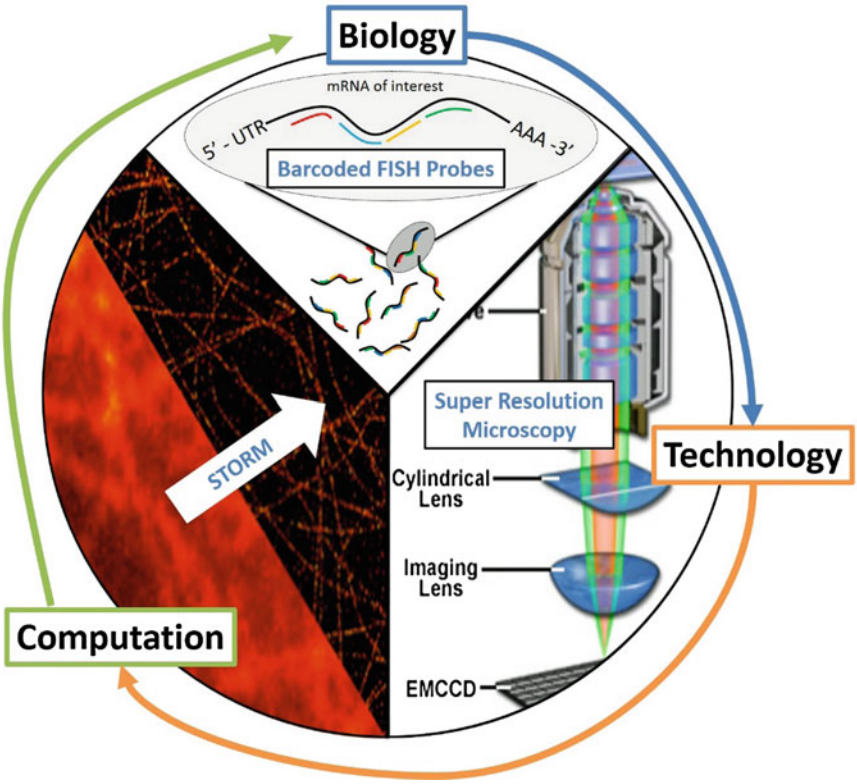

Fig. 6 Multiple modelling of mRNA transcripts as demonstrated by Lubeck et al., but displayed as an exemplary paradigm for the multi-disciplinary nature inherent to successful single cell systems biology. Biological questions are addressed by integrating new chemistries that are detectable using application of high-end technology. Novel computational strategies are devised to model the data acquired from the technology, which shows quantifiable data that loops back to inform new biological questions

concentrations of calcium. Intriguingly, should another colour be added to the barcode palate, in theory up to 792 mRNAs could be concurrently tracked in a single cell (Fig. 6).

4 Optical Detection and Imaging Systems for Single Cell Biology Systems

The final stage concerning single cells for use in systems level studies is a reliable detection/readout method for the resulting outputs of the single cell study. Taking advantage of the optical transparency of cells, light microscopy uniquely provides

non-invasive, 3D imaging of the cell interior. Moreover, specific cellular constituents, such as proteins, nucleic acids, and lipids, can be detected. Developments in the area of lens-based microscopy during the past 15 years have provided methods for investigating life at the subcellular level, discerning details below a quarter of a micrometre [27].

In addition to microscopic-based methods, various flow cytometry, image cytometry, and haematology instruments are also available for analysing cell populations [28, 29]. Typically, a platform is specifically adapted to the field of application, such as immunology, cancer biology, or microbiology. Ideally, these platforms should enhance the understanding of systems biology, across various physiological conditions.

4.1 Lens-Based Microscopic Methods for Single Cell Biology Systems

Numerous concepts have been utilised to "push and break the diffraction barrier" by reducing the focal spot size on lens-based microscopy systems. Single-point systems such as confocal microscopy, 4Pi microscopy and stimulated emission depletion (STED) methods have all become highly utilised in the area of single-cell biology systems. Parallelised systems combining numerous microscopic techniques have also emerged under the general term of reversible saturable, optically linear fluorescence transitions (RESOLFT), which can consist of systems based on STED, ground state depletion (GSD) microscopy, saturated pattern excitation microscopy (SPEM), saturated structured illumination microscopy (SSIM), photo activatable localization microscopy(PALM) and stochastic optical reconstruction microscopy (STORM). A comprehensive review of all of the above microscopic techniques has been presented by Hell [29]. The schemes are illustrated in Fig. 7 and comparisons of single-cell-resolution techniques are outlined in Fig. 8a.

Numerous applications using variations of the microscopy methods described above have been reported in recent years. Rust et al. have reported a high-resolution fluorescence microscopy technique based on STORM principles and the use of photo-switchable fluorophores [30]. The system reports an imaging resolution of 20 nm, which would be ideally suited for imaging single cells and sub-regions of single cells. Dempsey et al. have further evaluated photo-switchable fluorophores and defined a low cross talk, four colour super-resolution imaging system that can be used for single cell biology systems [31]. Schermelleh et al. have presented a comprehensive guide to super-resolution fluorescence microscopy and examples of the capability of such systems in the area of single cell biology systems are illustrated in Fig. 8b [32].

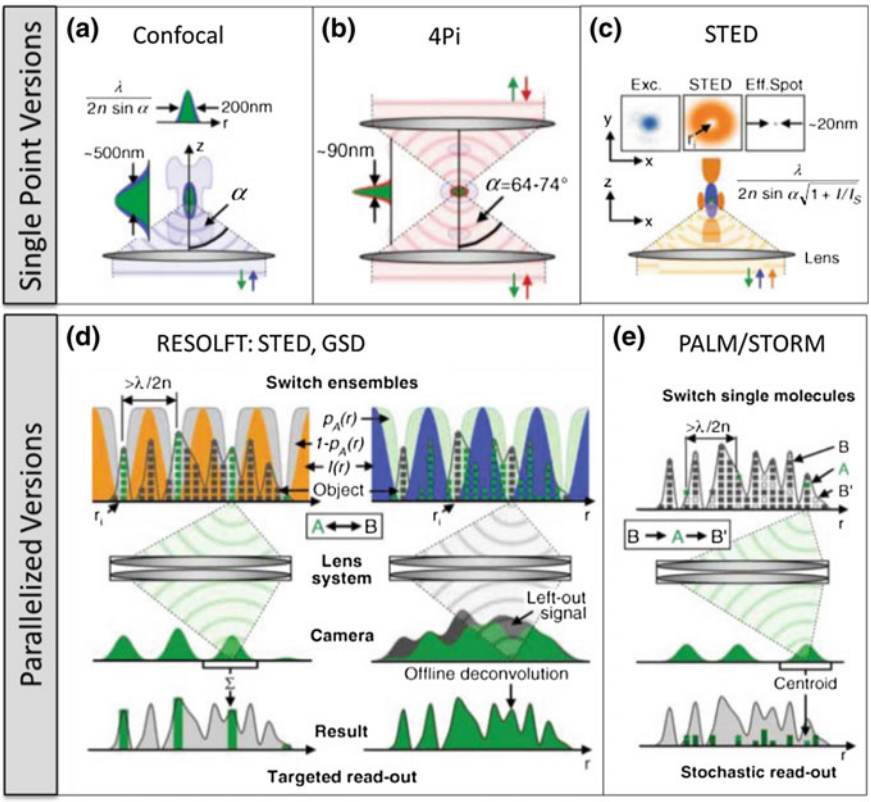

Fig. 7 Fluorescence nanoscopy schemes: single-point scanning (*upper row*) and parallelised versions (*lower row*). **a** Confocal microscopy. **b** By combining the wave front caps of two opposing lenses, 4Pi microscopy produces a narrower spot along the z-axis and hence an improved z resolution of 80–150 nm. **c** A typical single-point scanning STED microscope. **d** RESOLFT principle, corresponding to a parallelized STED, GSD, or photo-switching approach (on the *left*) and to the SPEM concept (on the *right*). **e** PALM and STORM read out the fluorophore molecules stochastically; the molecules must be switchable. From Hell et al. Ref. [27]. Adapted with permission from AAAS

4.2 *Cytometry-Based Methods for Single Cell Biology Systems*

Whilst they have found numerous applications in cell population analysis, ideal cytometry methods for single cell biology methods would incorporate imaging techniques (to obtain information on cell morphology) and absolute cell counts (similar to haematology systems).

Kantor et al. have reported a system where, in contrast to standard flow cytometry methods, the laser scans over stationary cells rather than cells flowing past the laser by using disposable capillary arrays instead of cytometry tubes or

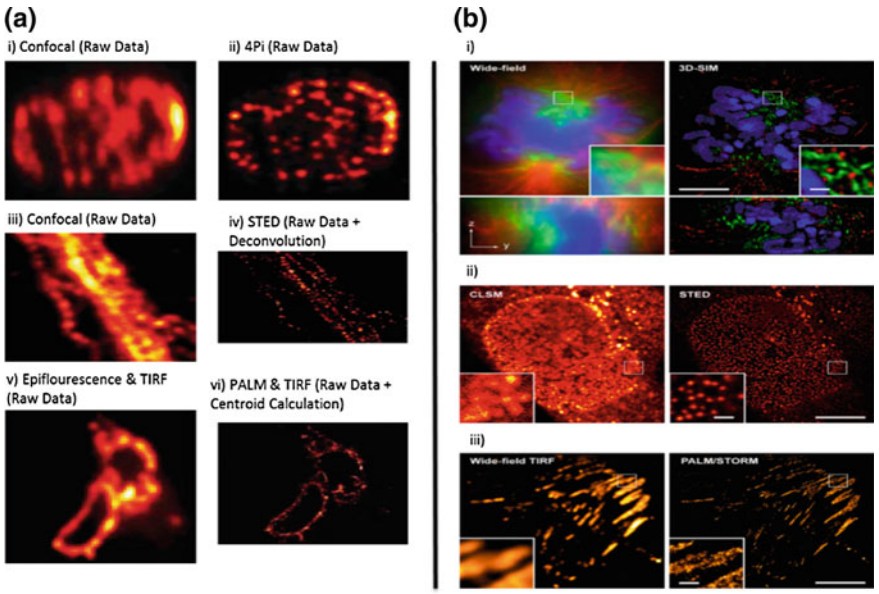

Fig. 8 **a** Comparison of single point and parallelised microscopy imaging for single cell biology systems. Images of microtubules in a neuron recorded with (i) confocal microscopy and (ii) 4Pi axial microscopy. Neurofilamentsin human neuroblatoma recorded with (iii) confocal microscopy and (iv) STED with nonlinear deconvolution. (v) Epifluorescence and (vi) PALM with centroid calculation, recording of a cryo prepared section from a mammalian cell (both images were recorded with a TIRF setup). **b** Super-resolution microscopy of biological samples. (i) Conventional wide-field image (*left*) and 3D-SIM image of a mouse C2C12 prometaphase cell stained with primary antibodies against lamin B and tubulin, and secondary antibodies conjugated to Alexa 488 (*green*) and Alexa 594 (*red*), respectively. Nuclear chromatin was stained with DAPI (*blue*). The bottom panel shows the respective orthogonal cross sections. (ii) HeLa cell stained with primary antibodies against the nuclear pore complex protein Nup153 and secondary antibodies conjugated with ATTO647 N. (iii) TdEosFP-paxillin expressed in a Hep G2 cell to label adhesion complexes at the lower surface. *Bars* 5 µm (insets, 0.5 µm). (**a**) From Hell et al. Ref. [27]. Adapted with permission from AAAS. (**b**) Reprinted from Ref. [32] Copyright 2010 The Rockefeller University Press

microscope slides [33]. A small cylindrical laser beam is scanned through the capillary in one direction while the capillary is translated relative to the optical detection system in a second, orthogonal direction. By scanning a predetermined volume for each capillary, absolute cell counts are obtained directly. In this respect, the system operates similar to haematology analysers [29], but different from laser scanning systems that focus on high-throughput screening [34], cell morphology [35], or rare cell [36] applications.

Over the past years numerous imaging cytometry systems have emerged, which integrate high-resolution microscopy imaging features into a flow cytometry instrument [8, 37] obtained from an imaging cytometry system is illustrated in Fig. 9, along with combinatorial imaging and fluorescence testing at single cell

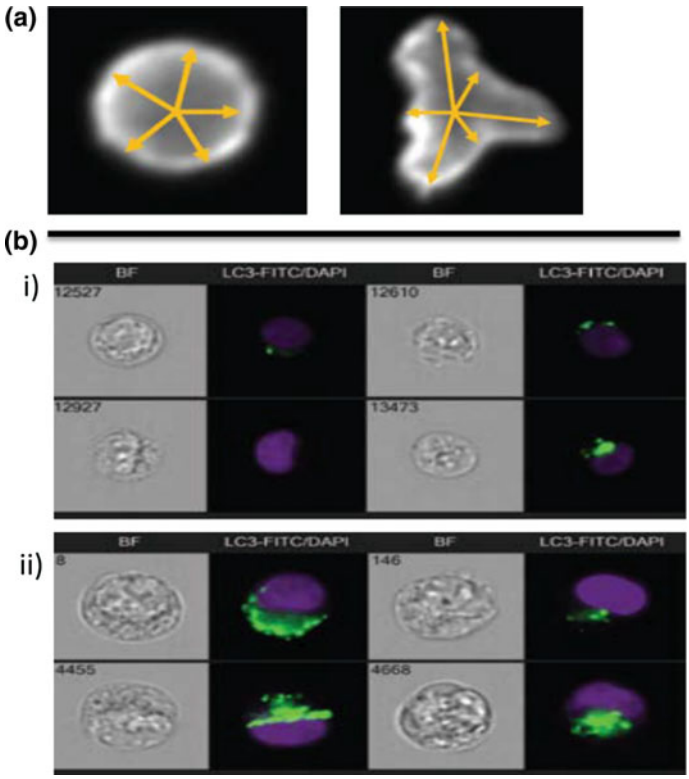

Fig. 9 **a** Cell morphology images for single primary monocytes having undergone drug treatment. **b** Representative bright-field images and merged florescence images for K562 cells in (i) control state and (ii) treated with etoposide to induce autophagy. Copyright Amnis Corporation, Seattle, WA, U.S.A Ref. [37]

resolution. Such systems can play a key role in single-cell biology systems as they can isolate single cells within a population whilst operating in a similar manner to a standard flow cytometer.

5 Conclusions and Outlook

The breadth of information produced in a modern biological science research laboratory is quite overwhelming when compared to that produced as recently as 10 years ago. Super-high throughput instrumentation, smart auto-targeting microscopy stages, imaging platforms capable of highly multiplexed optical investigations, and novel chemistries have all contributed to placing a vast amount of data into the hands of a researcher from even a single experiment. The age of "Big Data"

has certainly transformed the landscape of biology and applied medicine. It has brought it from a point where the identification of a single gene or epigenetic marker could send waves of excitement throughout the community, to where a complex assembly of interdependent cellular events can be elucidated by a single researcher; and where an entire genome can be sequenced overnight. It is rare that a researcher (or group) will have the full complement of staff with the necessary skills to (a) efficiently design a novel and massive throughput experiment, (b) develop novel instruments to carry out the experiment, and (c) mathematically analyse the considerable amount of data that ensues. The systems biology community has provided a framework whereby affiliation between groups with these skills can meet with a common goal, rather than each group seeking to acquire the skills in-house. This has allowed biological progress to keep up with the rapid expansion of capabilities in the mathematical and engineering sciences.

Single cell studies represent an area where systems biology can be applauded as a prime enabling strategy to advance cell biology to a new level. Single cell investigations have existed before the systems approach, and conversely systems biology has successfully been applied to multi-cellular studies. However, by illuminating and quantifying the rare but highly complex biochemical events that occur within an individual cell in a large population, combining the two has allowed new insights into the genesis of cellular diseases such as cancer. Population-based studies will often miss or marginalise important rare events, while non-systems level studies can focus too minutely on components of a defective biochemical system and hence miss the subtlety that leads to disease. Not all studies will require a systems level approach, or single cell resolution, but those that do now have the tools and framework to help steer biology and medicine to new and exciting insights.

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