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Cytomics and cellular informatics – coping with asymmetry and heterogeneity in biological systems

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Cytomics has a practical role to play in drug discovery within the immediate limitations of cell-based analyses. A more elusive goal is the clarification of the complexity of a dynamic cellular response to a drug through mathematical modelling. The common aim for drug discovery and cytomics is to bridge the molecular–cellular systems gap. The strategic challenges faced include: suitable preclinical biological models, cytometric platforms that resolve cellular or behavioural identity, analysis and visualization tools that act as integrating principles, consensus on standards and, finally, the implementation of informatics to serve the demands of decision making. Advances in all these key areas will help to address the need to identify and evaluate the origins of asymmetry and heterogeneity in cellular systems and thus reveal new opportunities for therapeutic targeting.

Cytomics overview

Cytomics is the study of complex and dynamic cellular systems starting with the single cell as its reference point. From this vantage position, molecular events within the boundaries of the cell can be explored together with a higher-level recognition of how cells populate, interact and behave within cellular networks [1,2]. The molecular profiling of bulk cell populations rapidly loses its resolving power and value once it is recognized that the cellular system under study is heterogeneous, displays asymmetric events or, perhaps more importantly, responds to a perturbing influence affects population dynamics [3]. This develops a cul-de-sac for many cell-based analyses in the drug discovery and development process. At one end of this problem, cytomics can reveal the 'informative cells' within a cell-based assay and clarify an endpoint readout using an expanding range of cytometric technologies of increasing throughput capacity [4]. At the other end, the large-scale acquisition and synthesis of multiparameter data from individual cells can provide an understanding of the behaviour of the system as a whole (i.e. the relevant cytome) and such data could be used to fuel predictive models in systems biology approaches. The implied existence of unique cytomes (e.g. a given cellular system, subsystems or functional component of the body) is not problematic because they are defined by the investigator in terms of limits of the heterogeneous cellular system of interest. Clearly, a cytome requires a more exacting definition of its 'boundaries' if it is to inform how molecular events might influence cellular dynamics and networks. This becomes more important as *in vitro* cell-based assays increase in the complexity of their components or when *in vivo* test systems are employed.

Cytomics has already broadened in its interpretation according to the community of interest. Cellular systems that pose challenges of increasing complexity include: heterogeneous microbial communities under changing microenvironmental conditions [5,6], normal physiology (stem cell dynamics, ion channel physiology, wound healing and senescence), neoplasia and constructed cell communities (tissue engineering) [7]. Cytometric approaches have also been used to explore the complexity of plant systems through analyses of various cellular metabolic and developmental characteristics (e.g. plant-specific cytoskeleton proteins [8]). Here, we focus on the cellular informatics challenge of cytomics relevant to the drug discovery arena, with particular reference to the properties of heterogeneity and asymmetry in complex cellular systems.

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Cytomics: emergence from cytometry and clinical decision making

Cytomics relies upon technical advances arising from various disciplines, not least cytometry [9]. Initial efforts in realising application areas for cytomics were focused on predictive medicine [10,11] arising from the single cell resolution power of flow cytometry and its pre-eminent position in clinical diagnostics [12]. The growing recognition of cytomics in the clinical setting was initiated by the availability of microarray gene expression technology for clinical sample analysis [13] and subsequently by the development of fully automated measurements of immunostained lymphocytes in tissue sections by means of digital colour microscopy – providing a virtual flow cytometry approach for dealing with cancer [14]. Furthermore, there have been attempts to exploit the underlying potential of the traditional perspectives of histochemistry and cytochemistry through efforts involving massive increases in the scale of data collection [15]. We can ask if cytomics approaches are likely to reach into complex cellular systems *in vivo*. A clue can be taken from multimodality imaging – rapidly becoming an essential approach, for example, in oncology diagnostics, tumour detection, staging and follow-up [16]. New technologies with imaging resolution at the cellular level in tissues show real promise for *in vivo* cytomics [17]. Furthermore, multimodality imaging, supported by the invention of functional contrast agents, should provide a reverse translational path of 'bedside to bench' to both inform and guide drug discovery efforts [18]. Here, the principle problem will be the interpretation of complex images in systems with a 3D context – ultimately an informatics challenge.

Cytomics: curbing enthusiasm

An ambition for cytomics in drug discovery is to bridge the molecular–cellular systems gap. The challenges faced include: (i) preclinical biological models that truly inform on drug effects; (ii) acquisition platforms that discern cellular or behavioural identity; (iii) analytical and modelling approaches that can act as integrating principles; (iv) and finally the development of informatics to serve the expanding demands of decision making in the discovery process [10,19]. The need for bioinformatics approaches for the extraction of knowledge from cytomics data sets is self-evident but this ambition begs for the standardization of experimental design and data [20]. Unfortunately, the challenge of developing models that can effectively integrate the molecular, cellular and organ levels for drug discovery purposes is considerable and certainly the span from pathway modelling to systemic disease biology is currently too wide [20]. Thus, current focus is on the long-term problem of extracting knowledge from the cellular and tissue level responses for bioactive agents.

Cytomics can have pragmatic aims for drug discovery, not least the removal of layers of complexity in a screen. For example, defining biological activity, as determined by cell-based assays, has been used to partition the chemical space for small-molecule screens in the identification of inhibitors of histone deacetylases [21]. Perhaps more importantly there is an increasing exploitation of intact live cells as reporters with their targets in context (see Ref. [22]). Subcellular localization and redistribution of proteins can be used detect responses within the structural and functional networks of normal and diseased cells, for example small-molecule high-content screens (HCSs) based on protein translocation assays have been used to yield hits for Akt1 translocation inhibitors, PI3K inhibitors, MKP-1 inhibitors, p38 mitogen-activated protein kinase (MAPK) inhibitors, activators of the wingless type/Frizzled (Wnt/Fzd) pathway and inhibitors of the p53–Hdm2 interaction [22]. Internalization and receptor activation assays in HCS mode of G-protein-coupled receptor (GPCR) assays have been used to screen for vasopressin V2 receptor agonists. Multiparametric hepatotoxicity HCS has exploited the availability of cell status-reporting cellular dyes [22]. Conversely, cytomics approaches can be used to search for informative phenotypes within complex populations to allow for the tracking of heterogeneity arising from asynchronous events (e.g. drug-induced cell cycle changes in checkpoint transition [23]). Target cell identification may also be important if molecular markers are carried by a subpopulation and those markers have linked phenotypes that may become asymmetrically inherited (e.g. cancer stem cells undergoing division and progenitor cell formation [24]).

Cytomics and the cell cycle

The cell cycle comprises a complex network of cell cycle proteins. In such an interaction network, proteins modulate one another's activities in a manner so complex that the dynamics of the system cannot be deduced accurately by intuitive reasoning alone [25]. Although cytomics provides an entry into biomedical cell systems biology [26,27], the linking of systems approaches at the molecular level to the cellular system is vital if predictive capacity is to be of real value [28]. Here, the translation gap is considerable because of the problems of data acquisition in providing informative single cell readouts and the mathematical challenges of encoding cellular responses. Breaking down the problem to cassettes that serve specific purposes in the study of drug action would seem reasonable. For example, advanced microscopy solutions already exist for monitoring the kinetics and dynamics of drug–DNA targeting in living cells [29] together with a linked mathematical model to describe intracellular micro-pharmacokinetics [30].

In the case of the cell cycle, a biological driver for drug discovery is to gain insight from mathematical modelling studies on how checkpoint activation and breaching resolve as perturbations in cell populations and thereby define a pharmacodynamic (PD) response. For example, cell cycle commitment in G1 and mitotic progression from G2 represent decision points for cells undergoing genomic stress signalling, with the problem of which bifurcation will take place under the influence of a checkpoint. Cytomics approaches to the real-time readout of such decisions has been enabled by the introduction of green fluorescent protein (GFP) sensors for cell cycle position suitable for HCS [23].

The considerable conservation of regulatory pathways from yeast to man has provided an impetus to the creation of cell cycle models [25]. The challenge for any theoretical cell cycle framework, however, is to integrate multiscalar parameters. Two-parameter bifurcation diagrams provide a theoretical solution for linking cell physiology (phenotypic events) with the protein activity and interactions (molecular events). The development of *in situ* imaging approaches to provide real data offers multiple challenges for the automated detection of subcellular patterns of proteins within cells [31] and at the same time defining a molecular event or state downstream of initial drug action – this is

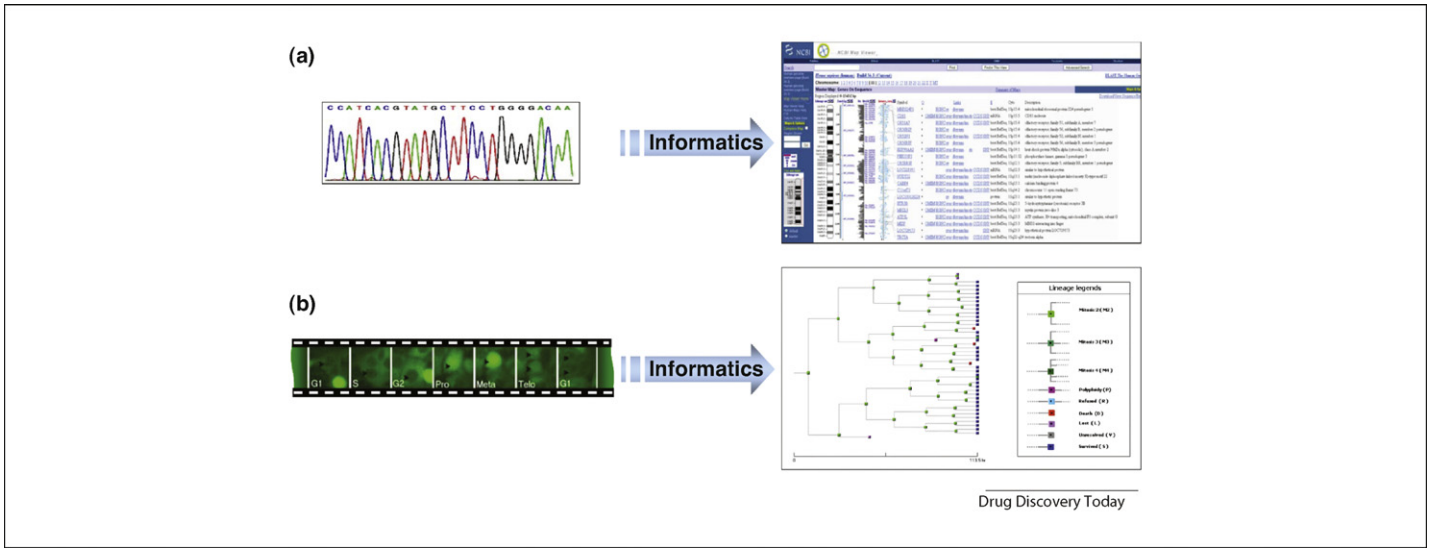

FIGURE 1

Visual representation of transformed 'omic' data. (a) Conversion of a lower DNA sequence organized to a given visualisation via the NCBI Map Viewer. (b) Conversion of a tracked single cell encoded from a microscopy timelapse sequence to a higher order lineage map to visualise cellular behaviour.

currently beyond reach for drug discovery exploitation and is likely to be limited by computational barriers [20].

Cytomics demands integrated data management strategies

The increasing scale of cytomics-based data acquisition is exemplified by the development of a highly integrated arrayed 234,000 microwell system to monitor the proliferative history of a large number of cells [28]. Recent advances have also made it feasible to conduct high-content small-molecule screens based on visual phenotypes of individual cells, using automated imaging and analysis [32]. Owing to the lack of connectivity and optimisation between different stages (e.g. from drug design, through image acquisition to data analysis), there are limitations for the systematic dissection or query of all the elements of a data management environment. Moreover, the development and application of new imaging tools will continue to demand data management adaptability [33]. Addressing such a generic challenge, Open Microscopy Environment (OME) [34] (see: http://www.openmicroscopy.org) was established to manage and access images alongside metadata that specifies the derivation of that image. A similar approach applies to flow cytometry data – MIFlowCyt [35] – reflecting a general move towards guidance on the coherent minimum information for reporting for biological and biomedical investigations (e.g. the MIBBI project [36]), the level of detail for the minimum information about a cellular assay (MIACA; http://miaca.sourceforge.net/) and the call for the development of standardized protocols to describe small-molecule screening [37].

Cellular informatics provides essential data structures for cytomics

While current large-scale projects address issues of cytometry data management, the next level of challenge is to extract information and convert cytometry data to knowledge [38], a critical step for establishing cytomics on a par with other 'omic' domains (Figure 1). There has been previous success in the application of software designed for transcriptomics to multiparameter flow cytometry with potential for the assessment of therapeutic intervention and for the association of disease-related biomarkers [39].

In drug screening applications it is likely that improved data visualization methods will be required for microscopy-based multicolour tissue cytometry [40]. A goal is to allow the various components of a cell to be visualized as they interact *in vivo* [41]. An ambition for the drug discovery community is to connect the nature and probability of a cellular response with the analysis of early molecular decision events – linking origins and outcomes often separated over wide timescales. Time-lapse imaging with linked cellular informatics provides a framework for establishing connectivity of events and has been used previously to track lineages and reveal the occult origins of drug resistance [42] [43] and the evasion of cell cycle checkpoints [44] (Figure 2). Developments in data acquisition platforms, analysis tools and predictive mathematical models have started to identify the need to deal with asymmetry at different levels.

Origins of asymmetry

Although a wide concept in biology, here we confine asymmetry to the accrual of differences between cell lineages (temporal asymmetry visualized as heterogeneity at a particular point in time) and the differences between the products of a given event (event asymmetry as demonstrated, for example, by stem cell division or the aneuploid products of a mitotic division under stress).

Temporal asymmetry as illustrated through a typical heatmap visualization (Figure 2), and associated cluster analysis can be translated as a PD response or drug signature. Such features can be exploited to predict the future behaviour of a cell population or to reveal occult drug signatures in a high-content screening environment, where the cellular behavioural pattern of a new chemical entity (NCE) is compared against known drug patterns. This last aspiration demands novel bioinformatics algorithms, similar to that of BLAST [45] used in genomics and proteomics.

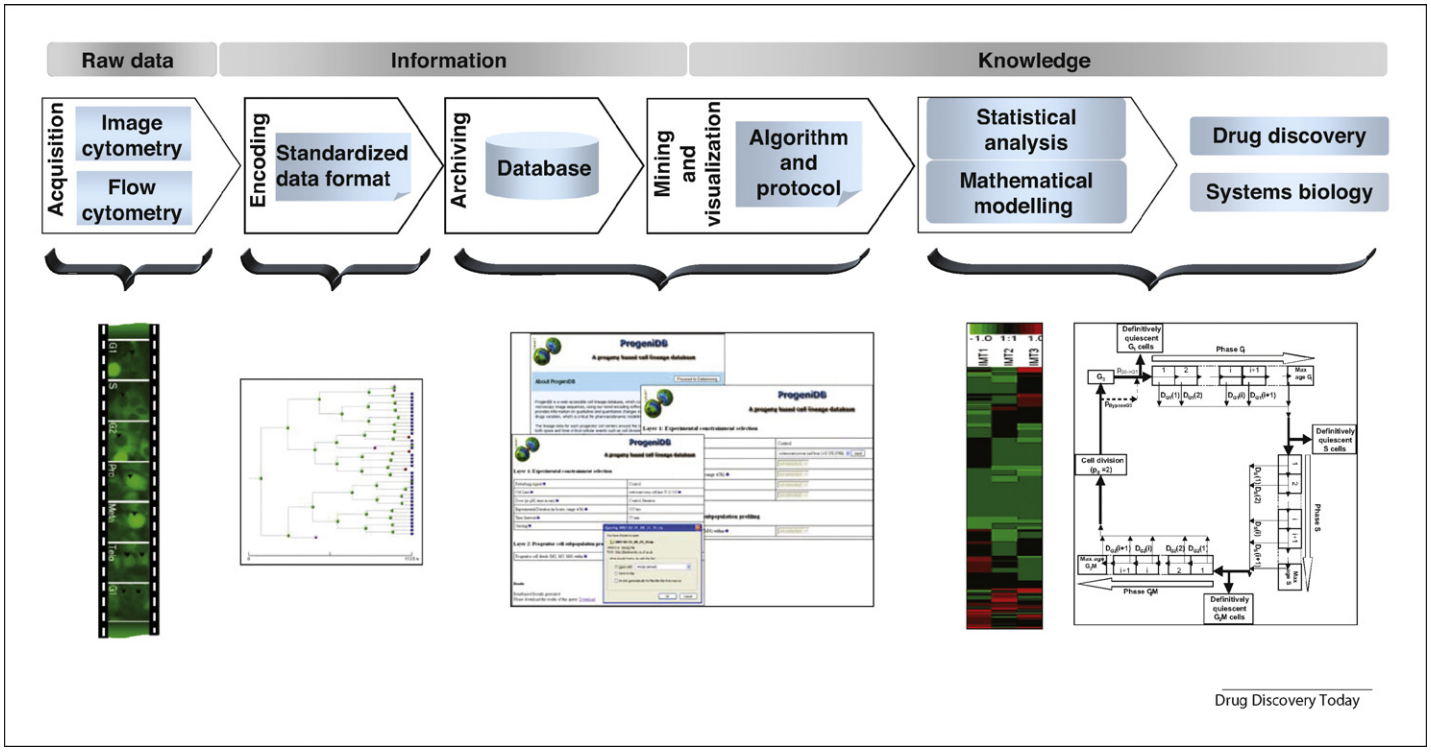

FIGURE 2

A typical cellular informatics environment required to transform cytometry outputs to reveal drug signatures. The top two rows represent an overview of infrastructure requirements and the bottom row illustrates some typical components of the infrastructure, such as: (i) acquisition – multiwell timelapse image sequence collection; (ii) encoding – data encoding software, through single cell tracking to transform raw image data into a standardized data format, for example the lineage map which provides metadata (information) about single cell behaviour; (iii) database archive – at this stage the raw data (i.e. images) may or may not be associated with the metadata, but the data format ensures the interoperability of the metadata and archiving ability within a standard database management system (e.g. MySQL, Oracle); (iv) data mining and visualization – are predominantly hypothesis driven and utilize specific algorithms and or protocols that, in turn, enable users to explore the metadata for new patterns and results; (v) statistical analysis and mathematical modeling – heatmaps and cluster analysis reveal cell cycle delay patterns, checkpoint breaching and drug resistance signatures and mathematical model validated by using the mined data. Such a cellular informatics infrastructure has been undertaken to achieve ProgeniDB [46].

Cell lineage formats have the potential to reveal asymmetric lineages for the descendents from a common progenitor during the evolution of a PD response [46]. The behaviour of the system, from the progenitor through the evolving progeny, reveals the time-integrated response to an influence such as a bioactive drug. This would therefore have a direct relevance to how cellular subpopulations, such as those with drug-resistant properties, might be maintained.

Temporal asymmetry may arise from the differential engagement of cell cycle checkpoints by a cell cycle targeting bioactive agent [42]. For example, intertwined DNA replication products generated by DNA topoisomerase II inhibition can lead to arrest in G2 through activation at a pre-mitotic decatenation checkpoint (DC) [47], providing a target for small-molecule modulator screens [48]. DC breaching serves as an origin for asynchrony. Abnormal mitotic events following DC failure can also result chromosomal instability (CIN) – providing an additional source of heterogeneity via event asymmetry at mitosis.

Various cellular structures are subject to asymmetric inheritance in normal and neoplastic cells with consequences for proliferation and survival capacity under various forms of stress. There are several precedents for this view. Aggresomes of misfolded proteins located at the microtubule organizing centre are generated by ubiquitin–proteasome system saturation. Asymmetric inheritance of aggresome cages of intermediate filaments or mitochondrial clusters allows one daughter to proliferate free of accumulated protein damage [49]. Asymmetric inheritance is influenced by the microtubule (MT) network [50]. It is known that the asymmetric division of stem cells requires tight control of spindle orientation [51] and, therefore, division will be influenced by cytoskeletal perturbations. Modelling the complex scenario of cells under MT stress, given the potential for asymmetric division and the temporal heterogeneity imposed by spindle checkpoint activation, may provide a therapeutic rationale for the combination of new proteasome inhibitors with histone deacetylase (HDAC) inhibitors [52]. Of course adult stem cells can divide asymmetrically to produce one self-renewed stem cell and one differentiating cell, maintaining both populations in a dynamic balance while the loss of polarity may also underpin tumourigenesis [53].

Asymmetric cell division: tracking and modelling

Cytomic approaches need to provide a parallel understanding of the divisional history of cells, for example through the modelling of [54] conventional carboxyfluorescein diacetate succinimidyl ester based cell tracking [55]. The real challenge, however, is to

dissect the impact and operation of asymmetric events, and the interrelationships of cells as lineages develop and perhaps collapse as part of a PD response. Engineered nanoparticles are increasingly providing advantages for the building of robust cell-based bioassays combining exceptional photo- and chemical-stability with other unique properties [56]. The potential for nanoparticle use in drug discovery, bioengineering and therapeutics has been reviewed previously [57]. Nanoparticle compartmentalization in endsomes [58] generates artificial aggresomes with implications for nanotoxicology modelling and the development of scalable methods for *in vivo* tracking and tumour profiling – a process driven by advances in chemistry and photophysics. Single-particle tracking is often the rate-limiting step in live-cell imaging studies of subcellular dynamics and there has been a recent description of approaches for dealing with high particle density, particle motion heterogeneity, temporary particle disappearance, and particle merging and perhaps most importantly splitting [59]. Immediate areas of application include the dissection of the mechanisms of receptor organization at the level of the plasma membrane. Quantum dot fluorophores provide a photo- and bio-stable optical marker signal well suited to the tracking of lineages within large cell populations over multiple generations. We have recently used a Monte Carlo algorithm to model the process of dot partitioning and dilution by cell mitosis. The application of genetic algorithms enables the simulation of cytometry data, revealing that the inheritance of the aggresome compartment occurs with a stochastic variation about an asymmetric split between each daughter cell after division.

Cytomics and drug discovery in the cancer stem cell cytome

Strategies for overcoming and avoiding the accrual of drug resistance in the treatment of cancer are constant themes in drug discovery and development. It has been suggested that, although key targets such as telomerase are currently leading the way to clinical proof-of-concept, the biology of senescence control and the regenerative characteristics of cancer cells with stem-cell-like phenotypes continue to drive research concepts vital to maintaining a clinical development pipeline [60]. In a stem cell compartment an enhanced expression of protective mechanisms against damaging xenobiotics or metabolites might be expected for an efficient maintenance of self-renewal properties. There is also a continuing drive to identify functional markers of neoplastic cells with stem-cell-like properties – cancer stem cells (CSCs) demonstrating a functional ability to sustain tumourigenesis and population heterogeneity through the 'stem-like' properties of asymmetric division providing self-renewal and differentiation capacities [24].

CSCs may contribute to tumourigenic and chemoresistant subfractions in a variety of malignancies, including brain tumours, leukemias and breast carcinomas [61,62], leading to a search for molecular targets for the specific elimination of CSCs [63] or identification of the mechanisms by which CSCs evade cytotoxic therapies [24]. Surprisingly, CSCs can be identified in subfractions of established cell lines [64–68] offering *in vitro* test systems for CSC-targeted agents and a demand for cytomics approaches that combine molecular markers and functional assays. Overexpression of the ABC half-transporter ABCG2 [69,70] can provide the cell with a means of efflux-mediated drug resistance and the transporter shows an intriguing linkage with the stem-cell-like phenotype. Enhanced transporter expression has been associated with the pluripotential side population (SP) of cells defined by their ability to exclude the DNA minor-groove-binding dye Hoechst 33342 [71–73]. Aldehyde dehydrogenase, an enzyme class that protects cells from the toxic effects of peroxidic aldehydes, also shows promise as a marker for the identification and isolation of stem cells from multiple sources [74,75], including human tumours [76]. A flow cytometric study has confirmed the presence of small subpopulations of cells excluding Hoechst dye in mouse retinoblastoma tumours (0.3%) and also that ABCG2/ALDH1A1-positive cells were Hoechst dye-dim [77]. Such crucial, but minority, target populations are clearly problematic for analysis and therefore require identification and characterisation rather than bulk population processing, given that such rare cells are often occult.

The wider context of multidrug resistance mediated by drug efflux pumps is under extensive investigation although the attempts to develop ABC transporter inhibitors as potential anticancer agents are yet to have real impact, given the limitations of first-generation agents, such as cyclosporine, and second-generation agents, such as valspodar and biricodar, resulting from their poor tolerability or interactions with other transporter proteins [78]. Third-generation inhibitors with high potency and specificity for P-glycoprotein (ABCB1) transporter may establish a true therapeutic potential for P-gp-mediated MDR reversal [78]. Nanoparticle therapeutics is also an emerging treatment modality for cancer because of the potential, via endocytic internalization, to bypass efflux via plasma membrane transporters [79]. Efflux reversal or bypass requires live cells for candidate agent screening and validation.

Concluding remarks: coping with increasing complexity

Attempts to improve the predictive value for drug discovery of cell-based disease models will arise from a capacity to deal with the molecular diversity of a human cytome [80]. A constant challenge is the establishment of suitable preclinical biological models to inform on drug effects in patients and designing systems that bridge this gap. Failure of preclinical screens to encompass the temporal and spatial aspects of biological systems limits the informed development of novel therapies [19]. High-throughput instrumentation, based around simple *in vitro* cell culture systems, is suitable for screening thousands of potentially active compounds but lacks context. *In vivo* micro-tumour systems have been used to recapitulate context – providing 3D tumour states – but translation to xenograft screens imposes high costs, long analysis times and inherently high variability requiring large numbers of repeat experiments. Solutions using micro-encapsulated cell systems [81] present new opportunities for cytomics approaches and cytometry. Future approaches, possibly involving refined optical methods such as ultrahigh-resolution optical coherence tomography [17] could be used to reconstruct cellular behaviour in such complex cellular systems.

As cytomics approaches are translated by different research communities [82] it is likely that wider-scale and generic hurdles will be recognized, requiring significant efforts in technology development [83] and a community consensus on standards. Perhaps, more importantly for drug discovery, a coherent

approach to the development of cellular informatics will allow the investigator to understand the impact that candidate agents have on new therapeutic targets and the off-target effects within complex cellular systems.

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